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14. ABSTRACT This grant focuses on identifying molecular features of prostatic adenocarcinomas (PrCa) from African Americans (AAs) which we hypothesize may differ from the molecular features of PrCas from European Americans (EAs). We give priority to characteristics found to be associated with PrCa aggressiveness, because the differential expression of those characteristics may, in part, explain why AAs tend to have more aggressive PrCa than EAs. Genes involved in fatty acid transport and metabolism have emerged as candidate markers for aggressive PrCa subtypes that are prevalent in AAs. We use an ancestry informed approach in which single nucleotide polymorphisms (SNPs) define more accurately the racial ancestry of our study subjects. AAs with PrCa are more often treated non-surgically and thus fewer radical prostatectomies are available to study PrCa in AAs. To overcome this disparity, this project focuses on the analysis of prostate biopsies. We use nitrocellulose blots (tissue prints) of prostate biopsies as a source of high quality RNA and DNA to identify molecular biomarkers that differ between AAs and EAs. These include PrCa generated field effects that modify adjacent prostate tissues even when they appear to be histopathologically normal; such field effects include epigenetic DNA hypermethylation with silencing of specific genes.

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INTRODUCTION

African Americans (AAs) have a greater incidence of prostate cancer (PrCa) than European Americans (EAs) and their PrCas tend to be more aggressive. Because aggressive prostate cancers are often treated non-surgically and because AAs frequently select radiation instead of surgery, there are disproportionately fewer radical prostatectomies available to study the molecular features of PrCas in AAs. Thus, much less is known concerning the biology of PrCa in AAs and this lack of knowledge can limit therapeutic options for AAs with PrCa, especially the choice of active surveillance (AS). To reduce this racial disparity in PrCa research in AAs, this project focuses on the molecular analysis of prostate biopsies in order to capture a more representative study population. We utilize an innovative tissue print technology in which nitrocellulose blots (tissue prints) are collected from each prostate biopsy core and used as a source of RNA, DNA and proteins for biomarker studies. By focusing on biopsies, we are able to identify molecular features of a wide range of PrCas including cancers from AA patients and from EA patients who select radiation therapy, have high Gleason scores and/or high stage of PrCas that cannot be successfully treated by radical surgery.

There are several hypotheses as to why PrCas are more aggressive in AAs including those considering social, cultural and economic issues that may delay evaluation of prostate health and appropriate screening and therapy for prostate diseases. Nevertheless, most studies have identified that biological issues also are likely involved in the aggressiveness of PrCas in AAs. One biological based hypothesis is that there are unidentified molecular characteristics that affect the biology of PrCas in AAs. These may be inherited genetic factors, DNA mutations in the tumor or epigenetic changes secondary to or interacting with other biological changes caused by, for example environmental exposures, diet, and/or obesity. To help differentiate inherited and environmental factors that may lead to more aggressive PrCa in AA, our molecular analyses include ancestry genotyping to identify West African, European (EU), and Native American (NA) ancestry based on single nucleotide polymorphisms (SNPs) that are used as ancestry informative markers (AIMs).

Our work during the last year revealed that self-identified AAs may fall into two subgroups that differ with respect to PrCa aggressiveness. Specifically, in a series of 83 self-identified AAs we observed that almost all (95%) of the AA individuals diagnosed with high grade PrCa (Gleason 7 or more) on biopsy had more than 75% West African (WA) ancestry by AIMs genotyping, while a large proportion (40%) of the AAs diagnosed with no cancer on biopsy showed more than 25% EU genetic admixture. This finding suggests that in AAs, ancestry genotyping may be helpful in assessing individual PrCa risk and provide useful information for AAs who are considering AS rather than immediate treatment. It also points to a need to adopt an "ancestry informed" approach to characterizing PrCa in AA populations.

Analysis of high grade cancers using prostate biopsy tissue prints has revealed prostate cancer subtypes that were either unrecognized or significantly underestimated in previous studies. Our gene expression data has identified the involvement of 3 molecules involved with the transport and/or synthesis of lipids that are highly overexpressed in a sub-set of PrCas. These include fatty acid binding protein 5 (FABP5) and fatty acid binding protein 1 (FABP1), genes that have not been previously reported to be differentially expressed in PrCas. Interestingly, higher levels of FABP5 expression seem to be more common in PrCas from AAs. A third lipid pathway gene overexpressed in sub-sets of PrCas is fatty acid synthase (FASN), previously shown to be involved in the emergence of castrate resistant PrCa. FABP5, FABP1 and FASN overexpression represent actionable alterations in genes that control PrCa lipid processing and metabolism that may reveal links between diet, obesity and aggressive forms prostate cancer.

During this reporting period, we obtained data by mass spectrometry as to proteins that macrodissected prostate glands from both AA and EA patients. These tissues also were analyzed for protein differences in PrCas between AAs and EAs. Comparing PrCas with uninvolved prostate glands, 53 proteins were identified to be statistically increased and 32 proteins were statically decreased. Comparing AAs with EAs, 10 proteins in AAs were statically increased and 21 proteins were statistically decreased.

In addition, in this reporting period, we have expanded observations of epigenetic effects of prostate cancer foci on surrounding uninvolved prostate glands, including promoter hypermethylatiion of genes such as (glutathione S-transferase 1) GSTP1, adenomatous polyposis coli (APC) and Ras association domain family member 1 (RASSF1). These are translatable as improved "field effect" tests that can be used to detect occult high grade cancer in patients who are considering active surveillance.

This reporting period also covers a significant expansion of our collaboration with the new MRI/US Fusion Guided Prostate Biopsy service at UAB. Because we can now overlay MR imaging information with pathology and with tissue-print molecular marker mapping, we are especially well positioned to translate what we are learning about PrCa in AAs to better guide decisions about active surveillance.

BODY

Administrative:

The major administrative problem involved the DOD action on a request from Drs. Gaston and Grizzle's laboratories for a no cost extension of their respective grants beyond May 31, 2015 (UAB) and June 2015 (Tufts). The no cost extension of Dr. Gaston was approved on 8/19/15. The no cost extension for Dr. Grizzle was approved on 9/15/15. Part of the problem with the delays was that UAB's Grants and Contracts Office had used the wrong grant number (actually Dr. Gaston's grant number) in yearly financial reports for Dr. Grizzle's grant. DOD did not report this to us until late in the administrative process. This period of administrative and financial uncertainty caused some shift in specific scientific approaches of the grant.

In this reporting period, other administrative issues included the renewal of IRB at UAB and the change in status of the UCA IRB from closed to active in order to collect additional information from patients who had been accrued at UCA. Because the collection of the contracted 60 cases of PrCa had been successfully completed at UCA in 2014, the Western IRB was asked to classify the IRB for UCA as being in a "data analysis" only category because no further cases were being accrued. In 2014, UCA without UAB's knowledge closed the UCA Western IRB and the UCA IRB at the DOD. Both IRB's had to be reopened for data analysis because additional data collection was needed for publication. This was completed and the additional data were collected and transferred to UAB. The IRB at UCA now remains in the "data analysis" category.

The Tufts Medical Center IRB has classified Dr. Gaston's component of this project as exempt.

During this period, UAB trained 4 student assistants, Ms. Fowler, Ms. Fuller, Ms. **Perez Aponte** and Ms. Sun. Others involved in the project did not change. During this period, Ms. Lian Tian was replaced as the technician in Dr. Gaston's laboratory at Tufts Medical Center with Mr. James Kearns. In addition, an undergraduate research intern, Mr. Ravi Chinsky, assisted with the project.

In 2015 Dr. Grizzle continued to be a consultant to a continuing grant DOD Prostate Cancer Tissue Repository. In addition, in 2015, he was added to the External Advisory Committee of this grant.

In 2015, Dr. Gaston DOD was chairperson for the March and July Special Emphasis Panels reviewing the NCI Innovative Molecular Analysis Technology SBIR grants (ZRG1 OTC-H (10), was an ad-hoc member of the NCI Chemo/Dietary Prevention Study Section in February (CDP) and a member of the NCI Cancer Detection, Diagnosis and Treatment Technologies for Global Health review panel in July (ZCA1 TCRB-6 (A1).

Specific Scientific Progress and Results:

Collection of Nitrocellulose Blots (Tissue Prints) for Analysis: In this reporting period, collection of tissue prints shifted completely to UAB with a focus on obtaining tissue prints from biopsy cores obtained using magnetic resonance imaging (MRI) fused with ultrasound (MRI-US) to guide the biopsy procedure. While biopsies obtained using the standard US approach are collected blindly as to areas of the prostate suspicious for cancer, MRI-US adds enough information from the MRI component to identify areas suspicious for cancer to which biopsies can be directed. In 2015, UAB accrued 15 patients (10 AAs and 5 EAs) from who biopsies were obtained using standard US technology and 53 patients (10 AA and 43 EA) from whom biopsies were obtained using MRI-US technology. In some cases, some biopsies are obtained only by MRI-US from areas suspicious for cancer while other biopsies are obtained from both areas suspicious for cancer as well as standard "blind" US biopsies. Tissue prints were obtained from all biopsy cores; overall a total of 835 tissues prints, 237 from AAs and 598 from EAs were obtained from all biopsy cores. This does not count tissue prints from one case which as discarded because of infection of the patient with Hepatitis C. Also, 13 tissue prints were obtained from two radical prostatectomies from patients with prior biopsies. These results are included in our summary of cases (Tables "EN"). A summary of our cumulative enrollment and biopsy tissue print collection is included in EN Tables 1-6.

Differential Expression of Prostate Biomarkers Associated with Lipid Transport, Syntheses, and metabolism.
Previously, Dr. Gaston used gene analysis of mRNA from blots of prostate cancer to identify the importance of fatty acid protein 5 (FABP5) in prostate cancer. This observation, confirmed by qrtPCR and by review of data published using gene sequencing studies identified a potential subset of PrCas which have elevated mRNAs for FABP5. Because of our focus on lipid transport, we elected to study two other related molecules associated with lipid control in PrCa including fatty acid binding protein 1 (FABP1) and fatty acid synthase (FASN). These molecules also were identified by qrtPCR to be elevated in PrCa. To evaluate the phenotypic differential distribution of FABP5, FABP1 and FASN in PrCa in AAs and EAs, UAB identified, collected, reviewed and selected paraffin blocks of normal prostate from radical cystectomy specimens from AAs and EAs and from AA and EA cases with radical prostectomies containing PrCa. Sections from immunostained cases were sent to Dr. Gaston who works with Dr. Kittles to identify racial admixtures. Also, for some immunostained cases, sections are sent to Dr. Gaston for qrtPCR analysis of biomarkers of interest.

Our studies in this reporting period have been focused on determining the differential expression of FABP 5, FABP 1 and FASN in patients evaluated for prostate cancers using radical prostectomies; also, we have evaluated these molecules in patients undergoing a radical cystectomy but found to have no prostate cancer in the associated removed prostates designated as (normal prostate)

We found that FABP5 is strongly expressed in prostate cancers but there is low to no phenotypic expression in normal prostate tissue or normal appearing (uninvolved) prostate glands from patients with prostate cancers. Higher values of FABP-5 were expressed in PrCas in AAs compared to EAs. There was expression of FABP5 in low and high grade prostate intraepithelial neoplasia (PIN). The intracellular pattern of expression in PrCa was primarily cytoplasmic with accentuation of staining in the areas of the cell membrane and areas of the nuclear membrane. There also was expression in the nuclei of PrCa cells. (Figure IHC-1, Figure IHC-2, Figure IHC-3 in drop box.)

In contrast to FABP-5, FABP1 had only slight to no differential expression in prostate cancers compared to normal prostate glands (no cancer) or to uninvolved prostate glands from the same matching cases of prostate cancer. Because of the large number of cases, differences between uninvolved prostate glands and PrCa is still significantly different at the cytoplasmic and membrane areas of the malignant cells. Of note, this low differential expression does not exclude FABP1 from being important as a potential target for therapy. The intracellular expression of FABP1 in PrCa has strong expression in the cytoplasm with somewhat stronger expression in the area of the cell membrane and the perinuclear area. There is weaker expression in the nuclei of tumor cells compared to cytoplasmic and membrane staining. (Figure IHC-4, Figure IHC-5). Figure IHC-6 in drop box).

Fatty acid synthase (FASN) has clear differential expression in prostate cancer when compared with the minimal expression in normal prostate glands (from non-cancer cases) and uninvolved (normal appearing) prostate glands from matching cases with cancer. In contrast to FABP5, the higher volume of FASN tends to occur in EAs. The intracellular expression of FASN is somewhat variable even for malignant cells within the same gland. In PrCa, there is prominent cytoplasmic and cellular membrane staining with accentuation in the perinuclear area. Of note, compared to FABP5, there is frequently no nuclear staining, in most cells of PrCa. However, in high grade tumors there seems to be an increase in FASN expression in nuclei. This change to an intracellular nuclear pattern may be an important regulatory pathway. (Figure IHC-7, Figure IHC-8), Figure IHC-9 in drop box).

Because FABP5 and FASN have been observed in some cases of PrCa to be inversely expressed at the mRNA level, we evaluated this at the protein level. This correlation is demonstrated for FABP5 versus FASN (Figure ICH-10), FABP1 vs FASN (Figure ICH-11) and FABP1 vs FABP5 (Figure ICH-12). The result for FABP5 vs FASN did not demonstrate the pattern observed at the mRNA level; however, the pattern did emphasize that there is increased expression in a subset of African Americans with a higher expression of FABP5 and a similar increase in expression for EAs of FASN (Figure ICH-10). This is demonstrated in TABLE IHC-1. Also, of interest, there is increased nuclear expression in subgroups of FABP5 and FASN which is indicative of a shift of FABP5 and FASN into the nuclei of some tumor cells. Because of the large ranges in expression of FABP5 and FASN, overall there is not a statistically significant difference in the overall pattern so the analysis is based upon cutoffs of phenotypic expression which vary with the expression in each of the intracellular areas (e.g. nuclear expression).

In summary, FABP5 is differentially expressed in patients with PrCa. Of these, patients with higher levels of PrCa, there is a predilection for these patients to be self-identified AAs. These results also were consistent with results of MS. In contrast, patients with higher levels of FASN tend to be self-identified EAs.

FABP5 and fatty acid binding protein 4 (FABP4) are increased in metabolic syndrome, which is a disorder which includes central obesity and elevated glucose and a tendency to develop cardiovascular disease and adult onset diabetes. The importance of metabolic syndrome has led to a widely used ELISA for FABP5 designed for serum. We have tested the FABP5 ELISA assay for FABP5 and found that it is technically reproducible and easy to perform; however, because of concern for the stability of multiple molecules in older samples of serum, (Potter et al 2012), we elected to postpone further evaluation of FABP5 in bodily fluids until a new set of fresh samples of serum are obtained. We have ordered these specimens from the Cooperative Human Tissue Network (CHTN) to facilitate our studies.

Discovery of Proteins in PrCa Using Mass Spectrometry: Our initial approach to identify molecules associated with the aggressiveness of PrCa and racial differences between these molecules used multiplex immune assays of serum, plasma and urine. A problem with these studies was that there were reports in the literature that specific molecules in bodily fluids began to change after 1 to 2 years (Potter et al 2012). Until we could address this problem, we shifted to mass spectrometry analysis comparing biomarkers in tissues of AA and EA patients, to identify molecules differentially expressed in PrCas. In this reporting period, using mass spectrometry (MS) we completed the analysis of 8 AA patients with prostate cancer and 12 EA patients without prostate cancer for discovery of proteins associated with self-identified AAs and self-identified EAs. For each category of patients, both paired PrCa and uninvolved prostate glands were macrodissected. Thus, this study identified proteins/peptides which are differentially expressed in PrCas in addition to proteins/peptides which are selectively overexpressed or underexpressed in AAs versus EAs.

The initial approach to mass spectrometry involved macrodissection of paraffin blocks of prostate cancer and matched uninvolved prostate from the same cases of prostate cancer. Thus a total of 20 specimens of macrodissected prostate cancer and 20 specimens of macrodissection matching uninvolved prostate were initially compared to identify molecules differentially expressed in prostate cancer. Each macrodissected specimen was evaluated in 3 dimensions (i.e., externally and longitudinally to ensure that no cancer was present in the specimen of uninvolved prostate glands and that the PrCas were composed of least 60% malignant cells). In these specimens a total of 896 proteins were identified and after these

were filtered and judged to be statistically relevant, 514 proteins were evaluated of which 53 were statistically increased in abundance and 32 were statistically decreased in abundance in PrCas with a false discovery rate of <0.1% (Figure MS1).

Based on systems analysis, the major organs and processes involved in the 85 proteins differentially expressed in PrCa are shown in Figures MS-2 and MS-3. The cytoplasm, extracellular proteins and nuclei were the most common tissue localizations identified as using the source of these proteins and cellular processes and cellular regulation were the most common themes in which these proteins were involved.

The most significant 20 proteins identified by their abundance (increased or decreased) to be differentially expressed are shown in Figure MS-4. The main proteins of interest are those which are **increased** significantly in PrCa.

After, the study of differentially expressed proteins in prostate cancer, we next focused on racial differences in prostate cancer, comparing PrCas from AAs with PrCas from EAs. We used the same approach to filter the 896 proteins to 298 proteins. Of these, 10 proteins were found to be statistically increased in abundance and 21 were found to be statistically decreased in abundance in AAs when compared to EAs. (Figure MS-5). The 10 proteins found to be increased in abundance are listed in Table MS-1 and those that are decreased are in Table MS 2

In view of our interest in lipid controlling molecules, we noted that zinc-alpha-2-glycoprotein, previously reported by others as a cancer marker that stimulates lipolysis is increased in PrCa in AAs. Because this molecule may be involved in the cachexia resulting from cancer, it will be added to our studies of lipids. Three other molecules of this group of 10 have been associated with motility and potential metastases. These are galectin-3-binding protein, alpha-actinin-4, and keratin, type II cytoskeletal 5. Also ubiquitin-like modifier-activating enzyme which has been proposed by others as a target for cancer therapy is elevated and is likely to be an important molecule in our study. Of interest and as expected, PSA is also increased in AAs compared to EAs. Several other molecules whose importance is unknown also are listed in Table MS-1 including SERPINA3 which will be discussed subsequently.

The 21 proteins that are decreased in tissue from PrCas in AAs versus Eas are listed in Table MS-2; however, our major focus will be on proteins that are increased rather than decreased unless important molecules that are decreased are identified in systems analysis. Figure MS-8 demonstrates the distribution in tissue of proteins in PrCa that are significantly changed. Specifically, cytoplasmic, extracellular and nuclear proteins are the most affected. Similarly in Figure MS-9 the main molecular functions of the significantly changed proteins involve binding, catalytic activity and structure of the tissue. The biological processes of the significantly changed proteins (Figure MS-10) involve cellular process and regulation. An example of changes in a specific protein (Fibrillin-1) is demonstrated in Figure MS-11 and a system analysis of SERPINA3 (ACT) is demonstrated in MS-12. Of note, SERPINA3 is a very important molecule in cellular responses to stress, control of immune responses and responses to cellular stimuli.

Evaluation by Mass Spectrometry of FABP-1, FABP-5, and FASN Based on Race: In the last quarterly report, we noted that FABP-1 was not detected by mass spectrometry; however, 7 cases had elevated FABP-5 in tumors with a normalized relative intensity (NRI) = 3.3 but no FABP-5 was detected in uninvolved prostate. Also, 15 cases had prostate cancer in which FASN was detected with a NRI = 4.6, but FASN was detected in only 2 cases of uninvolved prostate with (NRI=2.0).

For FABP-5, 3 of the 7 cases in which FABP-5 was detected were in AAs (3/8) with an NRI = 6.6 and 4 of the 7 cases were in EAs (4/11) with an NRI = 1.2 note one case of the twelve EA patients was lost to analysis. These results indicate that FABP-5 is more strongly expressed in prostate cancer from AAs than in prostate cancers from EAs and are consistent with results based on analysis at the mRNA level and with immunohistochemistry.

When FASN results were separated based on self-identified race, 7 of the 15 cases of FASN were in AAs (7/8) with and NRI = 4.4 and 8 cases were in EAs (8/11) with a NRI = 4.8. The two cases in which FASN expression was detected in uninvolved tissue also were in EAs (2/11) with a NRI = 2.

The 21 proteins that were decreased in specimens of prostate cancer from AA patients are listed in Table 2. We are still in the process of evaluating the potential importance of the decreases in each of these specific proteins on the aggressiveness of prostate cancers; however increased proteins are of major interest.

Ancestry Genotyping

When patients of different racial groups are analyzed and compared, the results can be affected by racial admixtures in the study populations which initially are not recognized. Dr. Rick Kittles, our collaborator on this DOD project, specializes in studies that incorporate ancestry genotyping into studies addressing health disparities. The Kittles lab analyzes DNA samples extracted from de-identified subjects based on single nucleotide polymorphisms (SNPs); he uses a profile of 109 unlinked autosomal SNPS that have been selected as ancestry informative markers (AIMs) to differentiate individuals of European, West African and Native American ancestry. In admixed populations, the AIMs results can then be used to estimate the relative proportion of these 3 racial groups in each individual's ancestry. During this reporting period, the Kittles lab completed ancestry genotyping for 126 of our study subjects, 114 from our prostate biopsy tissue print series and 12 from our radical prostatectomy (FFPE samples) series. The results of this analysis were provided to Dr. Gaston who has correlated the racial admixture results with the biopsy and tissue print results.

The overall pattern of racial admixture in our Birmingham area study subjects is similar to what has been observed in other US populations. As expected, many self-identified African Americans showed genetic evidence of ancestry admixture; in our Birmingham AA subjects this admixture was almost entirely from European ancestors with only rare individuals showing appreciable Native American ancestry.

The AIMs results from our prospectively enrolled prostate biopsy study subjects were particularly interesting. In our prospective study, ancestry genotyping was performed on DNA prints collected prior to biopsy and AIMs genotyping was performed by the Kittles lab blinded to both self-identified ancestry and biopsy results. Comparison of AIMs genotypes and biopsy pathology findings for the 83 self-identified AA subjects in the prospective biopsy study showed that, as a group, the men who were diagnosed with high grade PrCa (Gleason sum 7 or more) were more likely to have genotype estimates of more than 0.75 West African Ancestry, as compared to the men who were diagnosed with no cancer (P = 0.001) (Figure 1, Figure 2, Table 1). A similar trend is observed in a comparison of West African Ancestry between the men diagnosed with high grade cancer (Gleason sum 7 or more) vs low grade cancer (Gleason sum 6). To our knowledge, no previous studies have evaluated the levels of West African ancestry within a self-identified African American population as a marker for relative risk of prostate cancer. It should be noted that our studies use an ancestry genotyping panel with a relatively small number of well-established ancestry informative markers, and that this type of molecular testing is relatively inexpensive. Thus confirmation of a significantly increased risk of prostate cancer (and potentially, of high grade prostate cancer) in African American men with relatively high levels of West African genetic ancestry could have immediate potential clinical applications for prostate cancer screening and active surveillance. These findings were first presented during this reporting period at an invited Minorities in Cancer Research Scientific Symposium entitled "Emerging Methodology and Tools for Understanding the Genetics of Cancer Disparities" at the 2015 Annual Meeting of the American Association for Cancer Research.

Activities planned for next quarter: We have submitted DNA from an additional 61 study subjects to the Kittles laboratory for AIMs ancestry analysis. The Kittles lab has indicated that the analysis of these samples should be completed in time for us to present an update of our findings at the November 2015 AACR conference on AACR Conference on the Science of Cancer Health Disparities.

Mrna Gene Expression Analysis

Affymetrix Whole Transcriptome mRNA Gene Expression Profiling and qrtPCR Confirmatory Analysis: In this study we are using the Affymetrix Human Whole Transcriptome 2.0 (HTA 2.0) array to identify genes involved in prostate cancer and then confirm genes of interest using quatitative rtPCR (qrtPCR) technology. The HTA 2.0 is currently the most comprehensive array for interrogating human transcript isoforms for expression profiling. In addition to gene-level detection, this array provides the necessary coverage and accuracy required to detect all know human transcript isoforms produced from a gene. The HTA 2.0 design utilizes multiple data sources to design and annotate the array are RefSeq, Ensembl, UCSC Known Genes, UCSC LincRNA transcripts and Broad Institute - Human Body Map lincRNAs and TUCP (transcripts of uncertain coding potential) catalog. As with most gene profiling techniques, the HTA 2.0 array performs best with high quality RNA and because we routinely prepare RNA from biopsy tissue prints with RINs better than 7 (total RNA per prostate biopsy print approximately 200 ng) we have been able to take full advantage of this technology.

In addition to conventional analysis of our prostate biopsy gene expression data, we have found an approach described by Gorlov et al (2014) to be highly productive. These authors observed that while a typical approach to analyzing tumor gene expression compares cancer to adjacent uninvolved tissue, an analysis of inter-individual tumor-to-tumor variation in gene expression can be a more efficient way to identify genes that are over or under expressed in a molecular subgroup. One important advantage to this type of tumor-to-tumor analysis is that it is not confounded by cancer-associated changes in adjacent normal-looking tissue (cancer "field effects"). Our pairwise analysis of tumor-to-tumor variation in biopsies from AA and EA patients with high grade prostate cancer has identified several robustly overexpressed "outlier" genes of interest. These include genes involved in fatty acid processing and metabolism. Most notably, we identified a set of AA PrCa with extremely high (over 10 fold) overexpression of fatty acid binding protein 5 (FABP5). Although FABP5 has not been a major focus of PrCa research, qrtPCR studies confirmed this "super over-expression" pattern in a PrCa subgroup and identified a second PrCa subgroup with high overexpression of fatty acid synthase (FASN). Since our last annual report, we have focused much of our effort in further characterizing these two previously unrecognized PrCa subtypes.

At the level of the biopsy core, approximately 15-10% of the high grade prostate cancers show outlier "super overexpression" of FABP5 mRNA at more than 10 fold over the baseline expression observed in benign prostate biopsies in patients diagnosed with no cancer. A similar 15-20% of high grade prostate biopsy cores show outlier "super overexpression of FASN mRNA at more than 10 fold over baseline expression in benign cores from cases with no cancer (GE Figure 1). Outlier super-overexpression of FABP1 is also observed, but is less prevalent in our high grade biopsy cores (about 8-10%). Comparison of same-core mRNA expression patterns shows that while some tumors show moderate overexpression of more than one of these three markers, super overexpression is observed in an "either-or" pattern. Tumors with top quartile levels of FASN do not show super overexpression of either of the binding proteins, and vice versa (GE Figure 2). This observation suggests that there are two different ways for prostate cancers to satisfy their increased demands for fatty acids, either by de-novo synthesis (FASN) or by increased uptake from the extracellular environment (FABPs). When we look at expression patterns at the level of the study subject and consider the highest expression in any core we see that the pattern consistent with that seen by IHC in an independent set of samples, with AA predominant in the FABP5 super overexpressors and EA predominant in the FASN super over expressors. Interesting some multi-focal cancers show both FABP5 and FASN overexpressing clones, perhaps showing synergy as one focus synthesizes new fatty acids (FASN overexpression) and the adjacent focus takes advantage the excess (FABP overexpression). If these observations are confirmed as we move forward, we may be able to identify patients whose prostate cancer can be selectively targeted by pharmacological or dietary interventions that target these two lipid processing/synthesis pathways.

Analysis Of Epigenetic Cancer Associated Changes In Dna Methylation Patterns:

Scientific Progress and Results: DNA extracted from prostate biopsy tissue print nitrocellulose blots has proven to be very well suited for studies of genes in uninvolved normal appearing prostate glands postulated to be hypermethylated by field

effects secondary to adjacent prostate cancers. These studies have shown that the level of promoter hypermethylation of the genes GSTP1, APC and RASSF1 in normal appearing tissue (field effect hypermethylation) is more intense when the prostate contains high grade cancer, compared to that found in a prostate with only low grade (Gleason 3+3) cancer. This finding is potentially important, because it shows that a relatively straightforward modification of a currently available clinical test may be useful in identifying patients who are considering active surveillance based on a biopsy that failed to detect a high grade prostate cancer due to sampling error. During this last reporting period, we identified cut-off values that optimize this prototype biomarker test for predicting a low risk of occult high grade cancer. This work was presented at the annual meeting of the American Urological Association in 2015. This biostatistical analysis completes the manuscript that is currently being prepared for submission to PLOS ONE.

Additional data analysis comparing our field effect results with studies performed at Johns Hopkins Hospital in Boston is currently in progress; preliminary results have been submitted as an abstract to be presented at the 2016 GU ASCO conference. In addition, with support from the UAB Cancer Center, we will undertake a pilot study of the DNA hypermethylation patterns in areas of the prostate that are "suspicious" for prostate cancer. As noted in the section on MRI guided prostate biopsy studies, comparisons of AA and EA patients show a trend in which AA patients are more likely to have MRI suspicious regions that are negative for cancer in subsequent biopsy. We will test the hypothesis that a molecular test for cancer field effects may help differentiate MRI suspicious regions that are contain a an occult prostate cancer that was missed due to biopsy sampling error from MRI suspicious regions that are truly false-positive.

FABP5 and FASN are two lead molecular markers for our future studies because they may identify PrCa subtypes that are differentially prevalent in AA and EA, potentially significant as alternative fatty acid phenotypes that can be targeted therapeutically and potentially visible by Multiparametric-MRI (MP-MRI), as a result of changes in tissue composition. Additional markers that are under evaluation based on Affymetrix gene discovery data include fatty acid binding protein 1 (FABP1), elongation of very long-chain fatty acid 2 (ELOVL2), neuropeptide Y (NPY) and VEGF A. Additional markers under evaluation based on Mass Spec gene discovery including zinc-alpha-2-glycoprotein a controller of lipolysis and hypothesized to be involved in cancer cachexia and galectin-3-binding proteins reported in other cancers to affect aggressiveness.

FUTURE DIRECTIONS

• We will focus on increasing the number of patients for whom we will obtain tissue prints of biopsies of the prostate. Most of the emphasis will be on patients who are undergoing MRI-US guided biopsies. These patients also will have standard US guided biopsies and tissue prints will be obtained on all biopsy cores. Of special importance will be tissue prints from AA patients. Our goal is to have a manuscript submitted on this research in November, 2015 (Drs. Grizzle and Gaston).

• We will analyze ancestry informative markers (AIMs) from tissue prints to characterize racial admixtures (Dr. Gaston and Dr. Kittles) and will analyze mRNAs from tissue prints for genes of focus-FABP5, FABP1, FASN and zinc-alpha-2-glycoprotein.

• We will analyze racial admixtures from paraffin sections of cases analyzed by immunohistochemistry (Drs. Gaston and Kittles).

• We will analyze mRNA gene expression patterns from paraffin sections of radical prostatectomy cases analyzed by immunohistochemistry and MS in order to more completely characterize mRNA-protein-histology correlations for the genes involved in lipid processing and metabolism, with a particular focus on the high-expression prostate cancer subtypes that we have identified in our analyses of the prostate biopsy tissue prints (Drs. Gaston and Grizzle).

• For our immunohistochemical study of FABP5, FABP1 and FASN, we will analyze additional cases with Gleason scores of 6, 4+3, and 8-10. These cases will be selected so that each GS group will have balanced racial representations. We also will increase our analysis of normal prostate specimens from radical cystectomies (Dr. Grizzle).

• If resources permit, we will establish ELISA and multiplex immunoassays using samples of serum which are less than 1 year old. The ELISA will focus on FABP5. The multiplex immunoassay will focus on our prior studies of molecules that are increased in multiplex assays and new molecules identified by MS (Dr. Grizzle).

• Our goal is to add immunohistochemistry of zinc-alpha-2-glycoprotein and galectin-3-binding protein to our MS results and submit a paper by November 2015 (Dr. Grizzle).

• We currently are preparing a manuscript on this work in which we will perform immuno histochemistry on zinc-alpha-2-glycognotein and galectin-3-binding protein to demonstrate variations with race.

KEY RESEARCH ACCOMPLISHMENTS

• We have found that self-identified AA's who are diagnosed with PrCas with Gleason scores of ≥ 7 on prostate biopsy have a higher proportion (95%) of individuals with Western African ancestry $\geq 75\%$ than do AAs with Gleason scores of 6 or with no PrCa on biopsy.

• We have identified that FABP5, and FASN are molecules that are strongly expressed in PrCas ($p<0.0001$ for both). Of these cases, FABP5 is selectively expressed in AAs and FASN is selectively expressed in EAs.

• We have identified the FABP1 is slightly overexpressed in most PrCas at the protein level, but at the mRNA levels is highly overexpressed statistically in a significant subset of patients with PrCa.

• By mass spectrometry we have identified 53 molecules that are overexpressed and 31 molecules that are under-expressed in PrCas. We have identified 10 molecules that are overexpressed in AAs compared to EAs and 21 molecules that are under-expressed. One of the 10 molecules over expressed in PrCas of AAs is zinc-alpha-2-glycoprotein involved in lipolysis and hypothesized to cause cachexia of cancer. Another molecule of interest in PrCas of AAs is galectin-3-binding protein that has been associated with aggressiveness in other cancers.

REPORTABLE OUTCOMES

1. The abstract "Limitations of the use of human prostate tissues in biomedical research" was presented by Dr. Grizzle at the Prostate Cancer Foundation 21st Annual Scientific Retreat, Carlsbad, CA, October 23, 2014.

2. The abstract "Performance of an epigenetic assay to predict prostate cancer aggressiveness: Comparing Gleason score and NCCN risk categories" was presented at the EAU Section of Urological Research (ESUR) meeting in October 9-11, 2014, Glasgow, Scotland.

3. Dr. Grizzle was one of 5 presenters (Barnes M, Bledsoe MJ, Dressler L, Grizzle WE, Russell-Einhorn M) and panel participants in the all-day pre-meeting conference "Contemporary Issues in Biobanking: Governance, Consent and Practical Approaches to Current Challenges." This proceeded the conference "Advancing Ethical Research" of the organization, Public Responsibility in Medicine and Research (PRIM&R), Baltimore, MD, December 4, 2014.

4. An abstract "DNA hypermethylation field effects: potential applications for detection of occult high grade prostate cancer" was presented at the 2015 Genitourinary Cancers Symposium of ASCO on February 26, 2015.

5. An invited oral presentation on "Tissue Print Technologies for the Preparation of High Quality Human Biospecimens" was presented by Dr. Gaston at the SELECTBIO Sample Preparation and Analysis Technologies conference in Boston, Massachusetts, March 2015.

6. The abstract "Epigenetic Assay Stratifies Prostate Cancer Patients' Risk" was presented by Dr. Gaston at the 2015 American Urological Association Annual Meeting in May 2015.

7. An invited podium (oral) presentation "The use of innovative prostate biopsy tissue print techniques for molecular genomic, epigenetic and gene expression studies" was given by Dr. Gaston at the Minorities in Cancer Research Scientific Symposium "Emerging Methodology and Tools for Understanding the Genetics of Cancer Disparities" at the 2015 Annual Meeting of the American Association for Cancer Research.

8. An invited oral presentation "The Use of Tissue Prints of Prostate Cancer Biopsies for the Analysis of Non-Resected Prostate Cancer" was presented by Dr. Gaston at the Illumina Key Opinion Leader Biobank Summit in Boston, MA, May 2015.

9. An invited oral presentation on "Tissue resources and the Association of Racial Admixtures and the Risk for High Grade Prostate Cancer" was presented by Dr. Grizzle at the Illumina Key Opinion Leader Biobank Summit in Boston, MA, May 2015.

10. Abstract accepted entitled "Improving the accuracy and diagnostic power of prostate biopsy for African American patients: the Birmingham Alabama Prostate Cancer (BAPrCa) Consortium" to be presented by Dr. Gaston at the Eighth AACR Conference on the Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved in Atlanta, GA, November 2015 (copy attached to this report)

11. Abstract presentation entitled "Combined DNA-Methylation Intensity and Clinical Risk Score Stratifies Patients for High-Grade Disease" at the EAU Section of Urological Research (ESUR) meeting in Nijmegen, The Netherlands, September 2015 (copy attached to this report).

12. Invited oral presentation entitled "Tissue print technologies: An innovative and practical approach to obtaining high quality research samples from biopsies and other challenging biospecimens" will be presented by Dr. Gaston at IIR's Biorepositories and Sample Management Summit in Boston, MA, October 2015 (copy of the abstract for this presentation attached to this report)

13. Invited submission of FY15-FY16 PCRP program materials describing this DOD sponsored project; these materials include text and images for the program booklet, CDMRP website features, and the PCRP newsletter (PCRP Perspectives). September 2015 (copy of the invitation is attached to this report).

14. Invitation to Dr Grizzle and Dr Gaston to apply for the DOD PCRP Health Disparity Research Award, Application submitted September 24 2015.

15. With our collaborator Dr. Soroush Rais-Bahrami as PI, Drs. Gaston and Grizzle are co-investigators on a newly awarded pilot grant from the UAB Cancer center to explore potential correlations between MRI parameters that define regions of the prostate as "suspicious" for prostate cancer and molecular prostate cancer field effects. September 2015

16. Book Chapter in Press
    Burke HB, Grizzle WE. Clinical Validation of Molecular Biomarkers in *Translational Medicine in Biomarkers in Cancer Screening and Early Detection*, Sudhir Srivastava, editor, Wiley, Oxford, UK.

CHALLENGES AND PROBLEMS

Our main challenge affects all research in prostate cancer. Specifically, it is difficult to define "aggressive" PrCas except by association with Gleason score because of the many indolent PrCas and the long time it takes to define a recurrence of these tumors. Thus, we rely on Gleason score to correlate with aggressiveness except for tumors that are known to reoccur. Similarly, when working with bodily fluids, the "controls" (i.e., cases without PrCa) are difficult to define because PrCas tend to be asymptomatic and cases biopsied may be false negatives in that the lesions may be missed on biopsy. This is one reason that we have begun to select cases biopsied by MRI-US. Also, we are still trying to define changes in bodily fluids that may occur in storage at $-80^{\circ c}$ or colder. We are addressing this by trying to analyze samples of bodily fluids that are $\leq$ 1year of age. We also will match samples by month of age. Our final challenge is trying to decide when to publish our positive results. We have now collected tissue prints from over 100 AAs and 80 EAs. We await the ancestry informative markers from more recent cases with a goal of publishing our initial manuscripts in November 2015. Other than associated with the above challenges, we have no major problems of which we are aware.

REFERENCES

Potter DM, Butterfield LH, Divito SJ, Sander CA, Kirkwood JM. Pitfalls in retrospective analyses of biomarkers: A case study with metastatic melanoma patients. J Immunol Methods 2012;376 (1-2):108-112.

Gorlov IP, Yang JY, Byun J, Logothetis C, Gorlova OY, Do KA, Amos C. How to get the most from microarray data: advice from reverse genomics. BMC Genomics. 2014;15:223.

SUPPORTING DATA

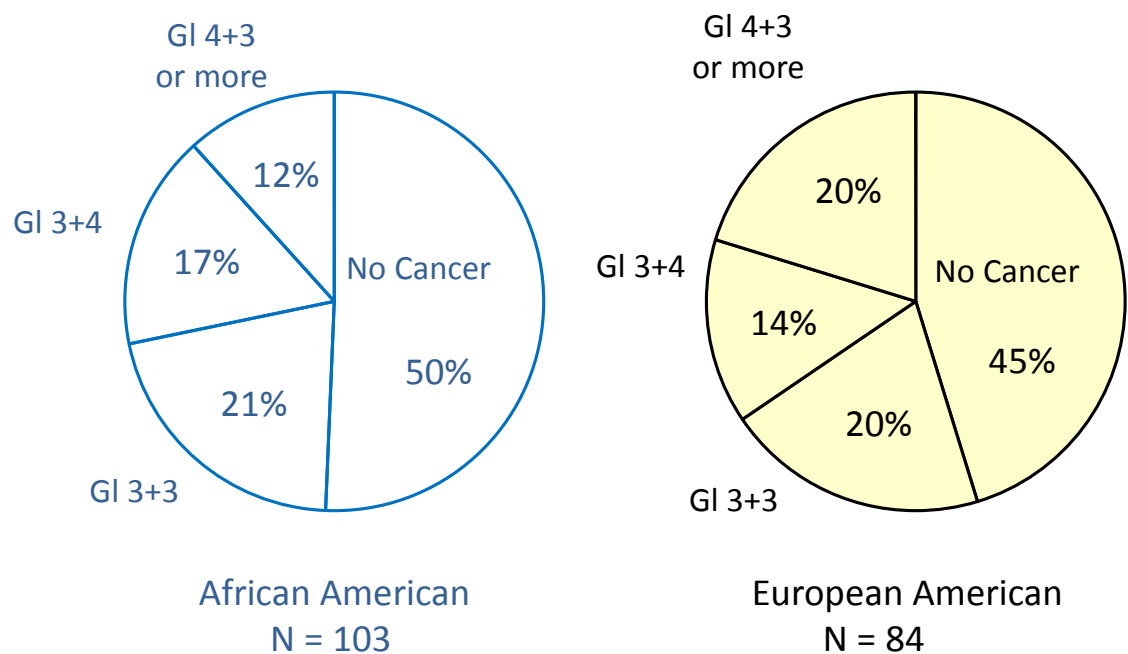| Study Subjects Enrolled and Biopsy Tissue Prints Collected*			
Tissue Prints Collected from UCA and UAB as of September 30, 2015			
	All races	AA	EA
All Subjects	187	103	84
Benign	90	52	38
High Grade	58	29	29
Low Grade	39	22	17

* Note that Enrollment Tables Exclude 2 Study Subjects
1 Withdrawal from UCA and 1 Omitted from UAB (both AA)

EN Table 1

Enrollment at UCA and UAB Study Sites*			
Tissue Prints Collected from UCA as of September 30, 2015			
	All races	AA	EA
All Subjects	59	59	0
Benign	33	33	0
High Grade	12	12	0
Low Grade	14	14	0

Tissue Prints Collected from UAB as of September 30, 2015			
	All races	AA	EA
All Subjects	128	44	84
Benign	57	19	38
High Grade	46	17	29
Low Grade	25	8	17

* Note that Enrollment Tables Exclude 2 Study Subjects
1 Withdrawal from UCA and 1 Omitted from UAB (both AA)

EN Table

Summary of All Study Subjects	African American		European American	
Diagnosis Group	N	% of 103	N	% of 84
All Subjects	103	100%	84	100%
No Cancer on Biopsy (Benign Diagnosis)	52	50%	38	45%
Ca Positive, Low Grade (Only Gl sum 6)	22	21%	17	20%
Ca Positive, Gl sum 3+4	17	17%	12	14%
Ca Positive, Gl sum 4+3 or more	12	12%	17	20%

Summary of Cancer Positive Study Subjects	African American		European American	
Diagnosis Group	N	% of 51	N	% of 46
Cancer Positive Subjects	51	100%	46	100%
Ca Positive, Low Grade (Only Gl sum 6)	22	43%	17	37%
Ca Positive, Gl sum 3+4	17	33%	12	26%
Ca Positive, Gl sum 4+3 or more	12	24%	17	37%

EN Table 3

EN Figure 1: Biopsy Diagnosis in AA vs EA Study Subjects

MRI Targeted Biopsy Study Subjects Enrolled as of September 30, 2015					
Diagnosis Group	All races	AA	% of 11	EA	% of 47
All Subjects	58	11	100%	47	100%
Benign	28	8	73%	20	43%
High Grade	19	1	9%	18	38%
Low Grade	11	2	18%	9	19%

EN Table 4

MRI Targeted Biopsy Subjects as of September 30, 2015 Comparison of Standard and Targeted Cores					
Diagnosis Group	Subjects	All Std Cores	All Tgt Cores	Low Gl Cores	High Gl Cores
All Subjects	58	420	324	50	64
Benign	28	181	155	0	0
High Grade	19	155	94	21	64
Low Grade	11	84	75	29	0

Conventional (non-MRI) Biopsy Subjects as of September 30, 2015				
Diagnosis Group	Subjects	Standard Cores	Low Gl Cores	High Gl Cores
All Subjects	129	1675	114	173
Benign	62	871	0	0
High Grade	39	468	47	173
Low Grade	28	336	67	0

EN Table 5 and 6
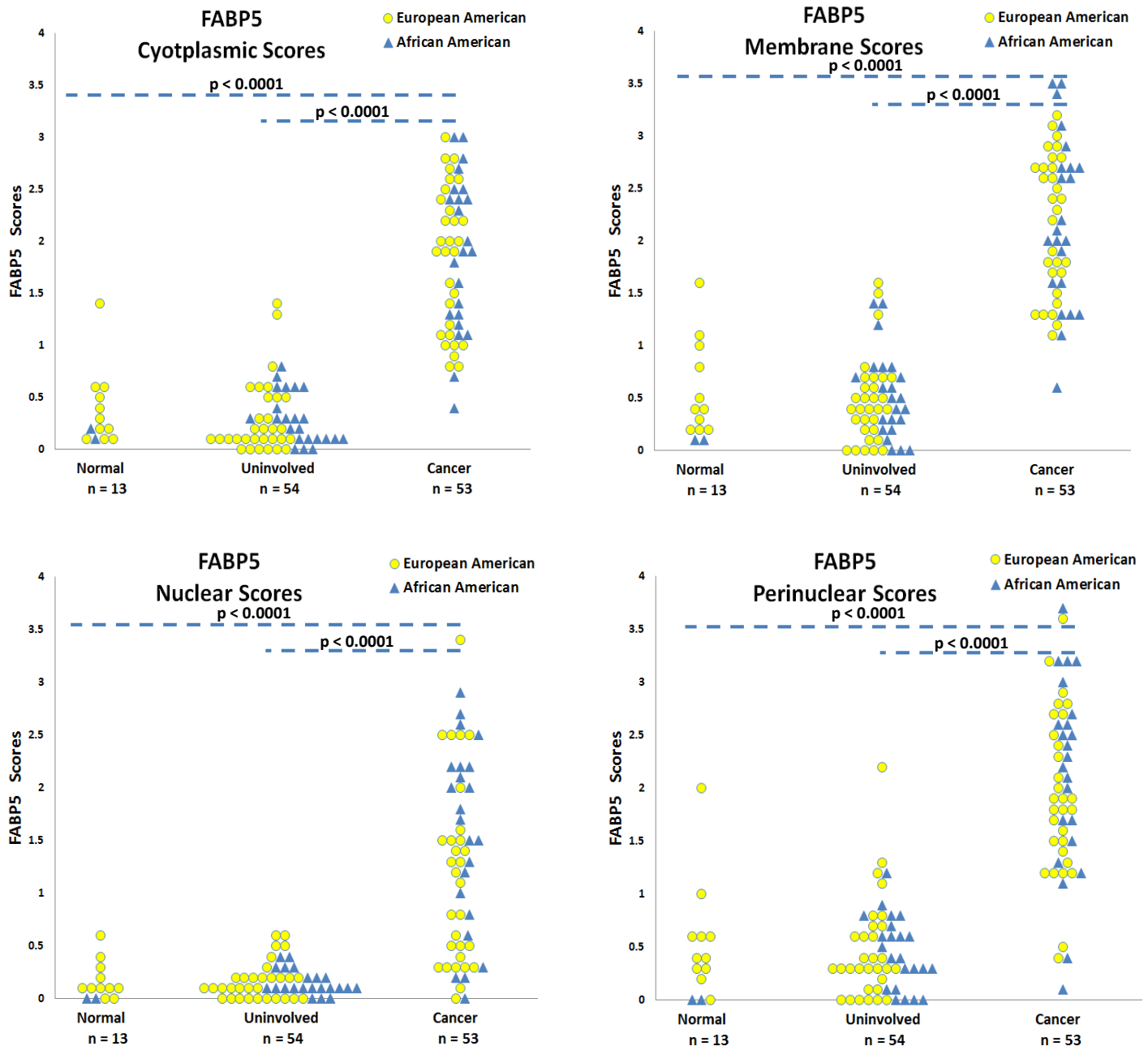
Legend

Figure IHC-1 Expression of FABP5 in the prostate. This figure demonstrates FABP5 staining that is broken down as to intracellular patterns of staining- cytoplasmic, membrane, nuclear and perinuclear. Normal is based on prostates removed during radical cystectomy. These prostates did not have PrCa on pathologic examination. Uninvolved represents the matching normal appearing prostate glands from case of prostate cancer. While "cancer" indicates the matching PrCa to the uninvolved glands. Note, there is statistically significant differences between PrCa (cancer) and normal (p< 0.0001) and PrCa and uninvolved glands (p< 0.0001). Of interest, in the high levels of expression of FABP5, there are somewhat more African Americans (Table IHC-1) in all categories of expression, but especially in nuclear expression.
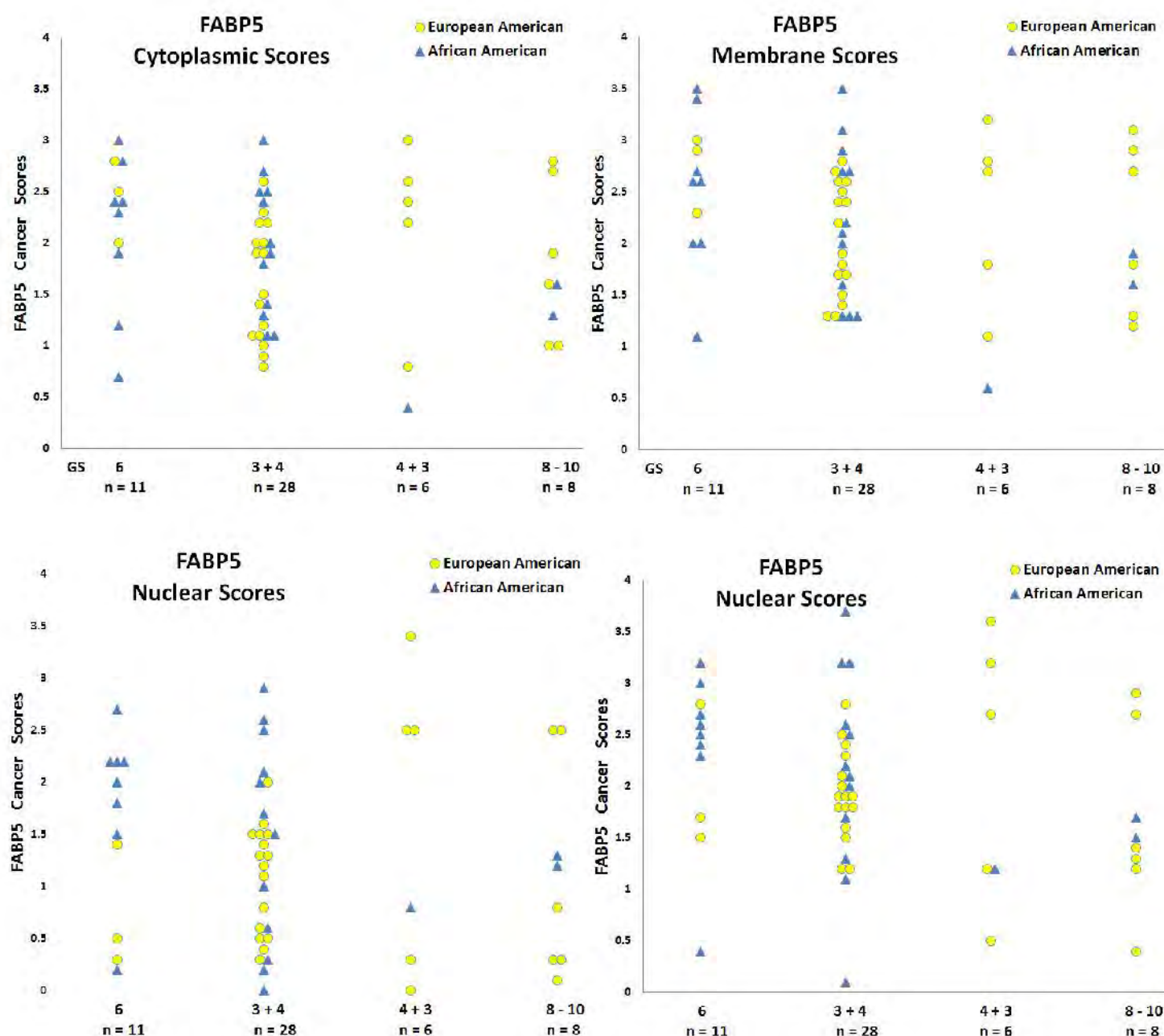
Legend
Figure IHC-2 Expression of FABP5 in PrCa. This figure demonstrates the intracellular distribution of staining of PrCa-cytoplasmic, membrane, nuclear and perinuclear. The PrCa is broken down according to the Gleason Score (GS) of the case, i.e., GS 6, GS 3+4, GS 4+3, and GS 8-10. These are the cases randomly selected to date. The number of cases with GS 6, 4+3, and 8-10 cases will be expanded in the next quarter.
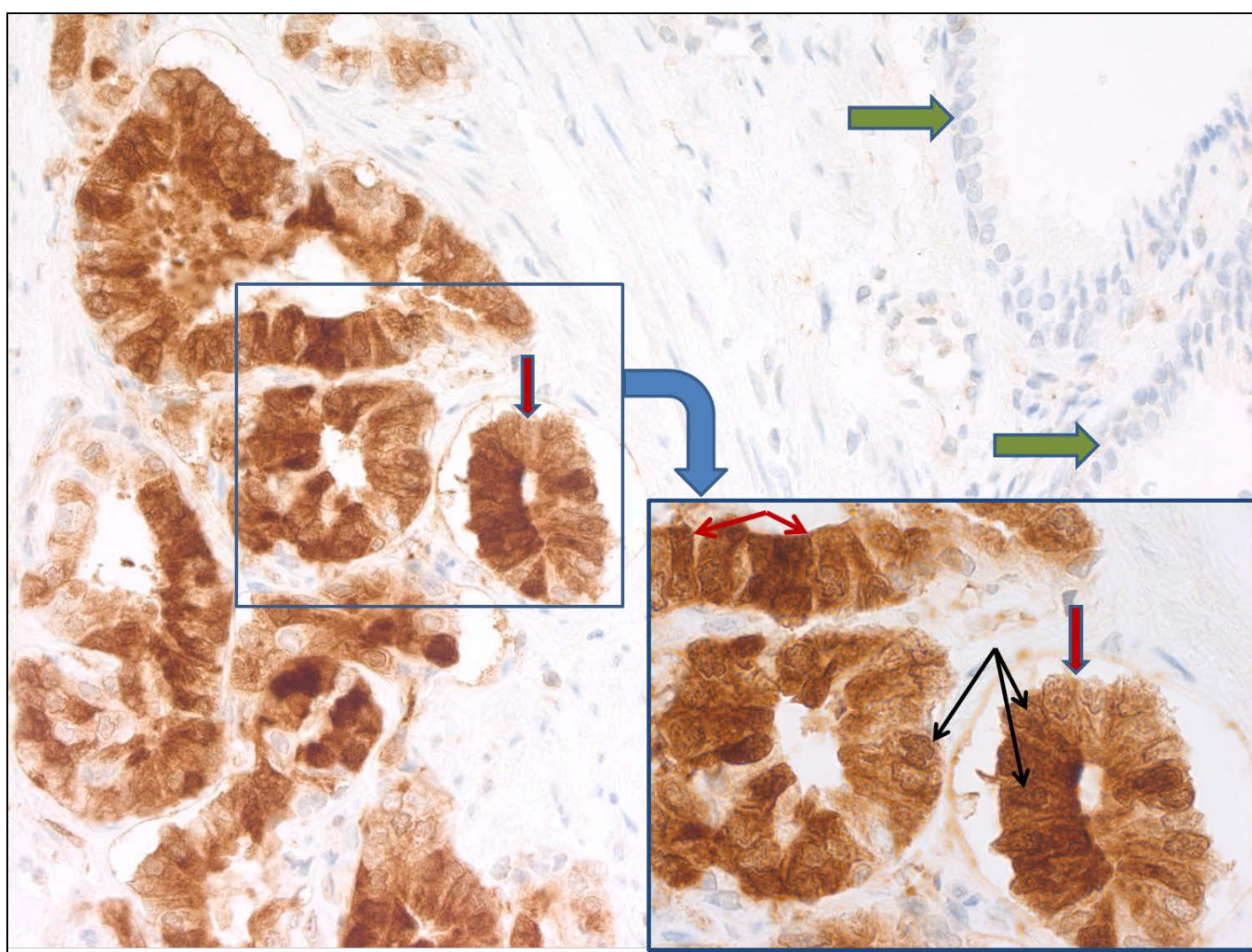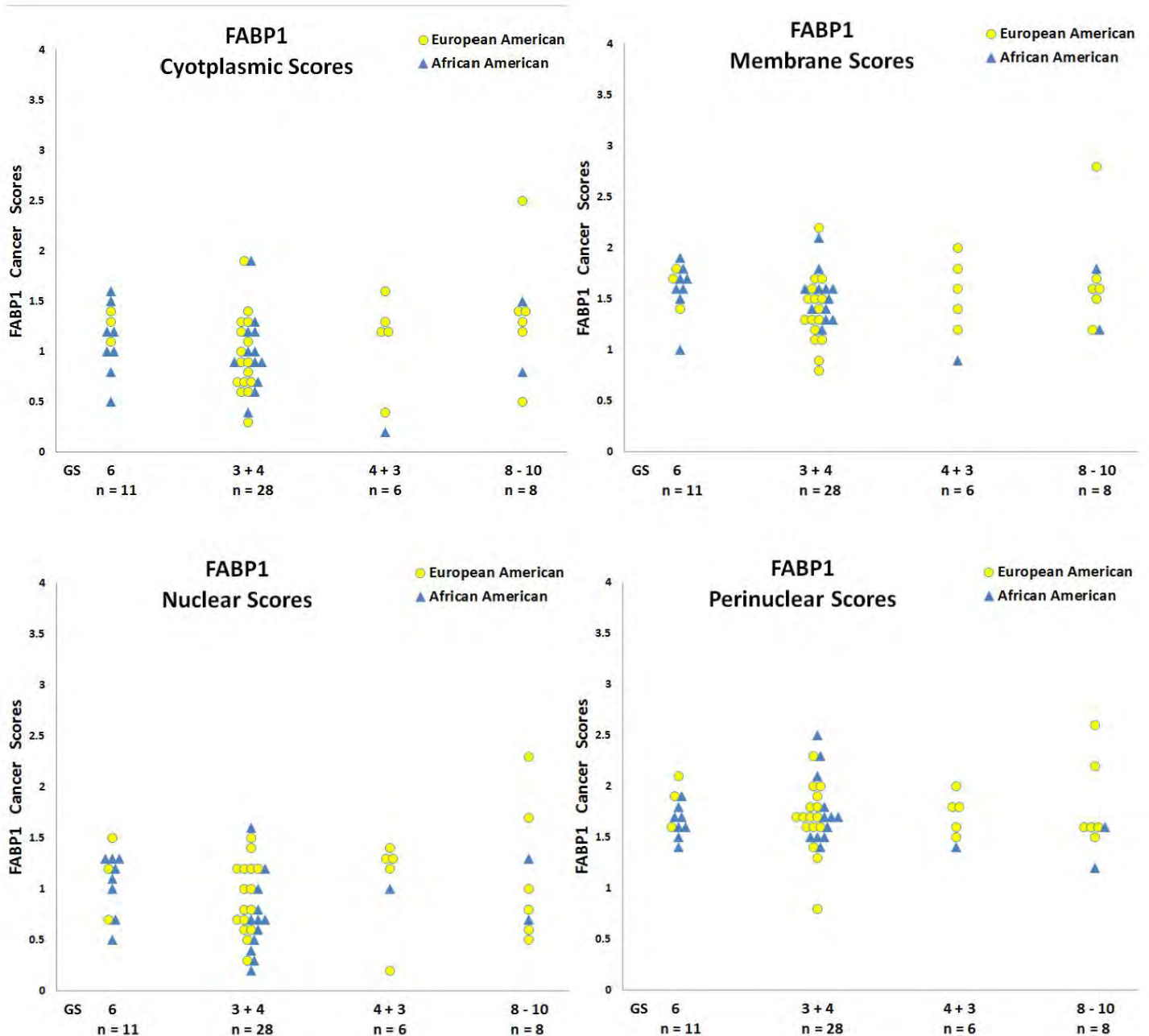
Legend:
Figure IHC-3 Expression of FABP5 in PrCa. This figure demonstrates the intracellular distributions of FABP5 in an area of prostate cancer (red block arrows) adjacent to uninvolved glands with minimal staining (green block arrows). Magnification x400 focally increases to x630. Thin black arrows point to nuclear staining with FABP5 and red arrows (thin and block) point to staining of the cell membrane.

There is variable staining of cells with FABP5 even within the same malignant gland and there is little to no staining in uninvolved glands. FABP5 staining is increased at the membranes of cells and in the nuclei of some cells. This is emphasized in figure IHC-1.
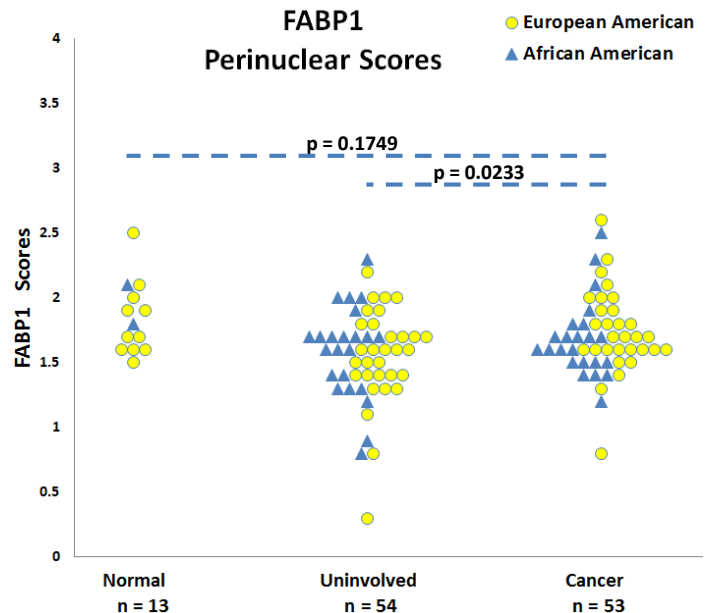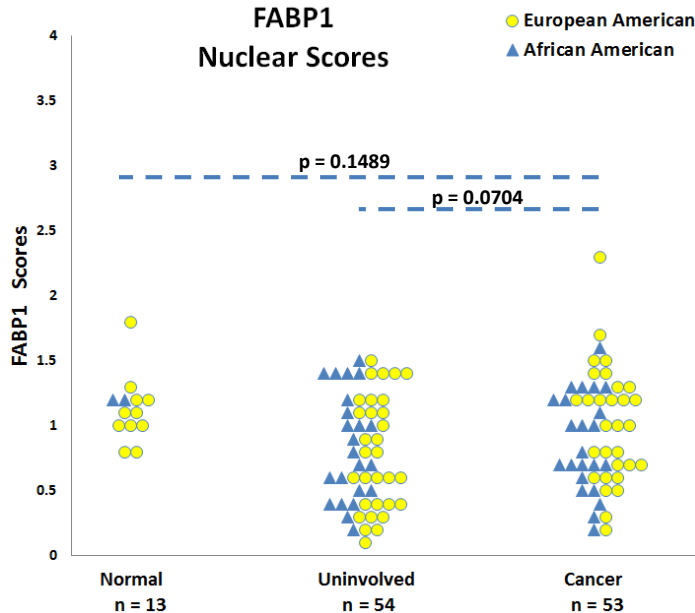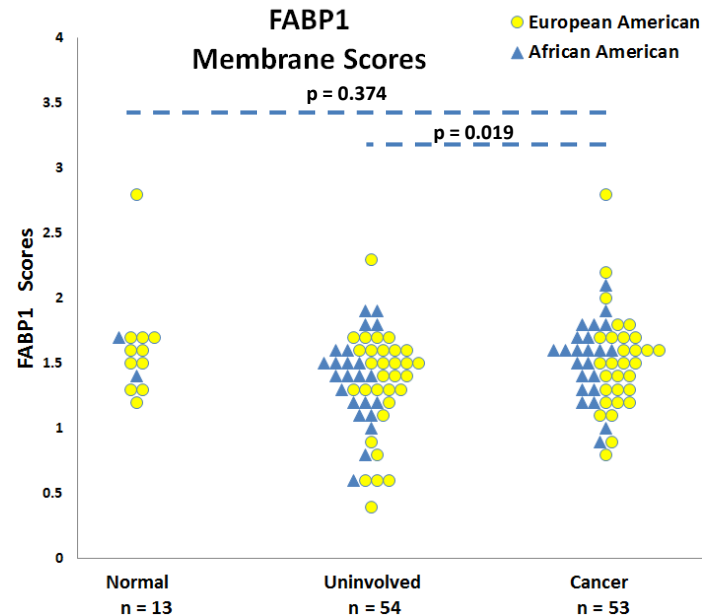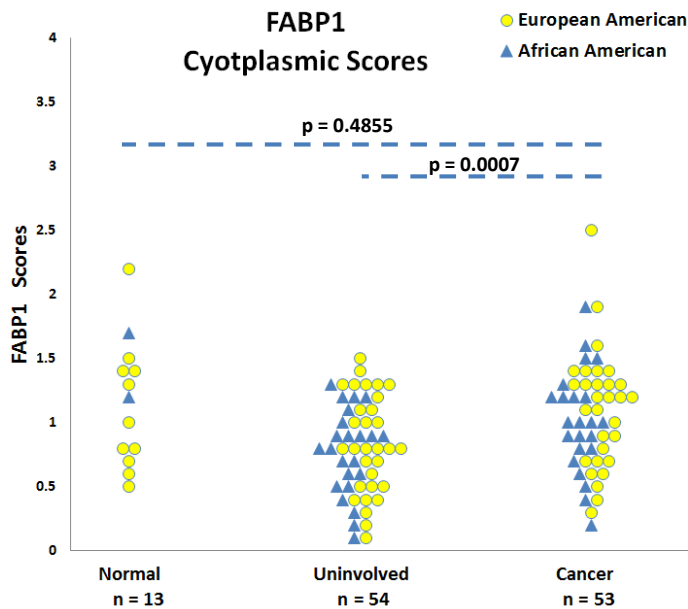
Legend

Figure IHC-4: Expressions of FABP1 in the prostate. This figure is similar to IHC-1 except that FABP1 is being evaluated. Of note, there is much less differential expression of FABP1 between the normal glands and PrCa and the normal appearing uninvolved glands and PrCa than there is for either FABP5 (Figure IHC-5) or FASN (Figure IHC-7). Although this degree of differential expression is small, for cytoplasmic expression and membrane expression when uninvolved glands are compared with PrCa there is a statistical difference
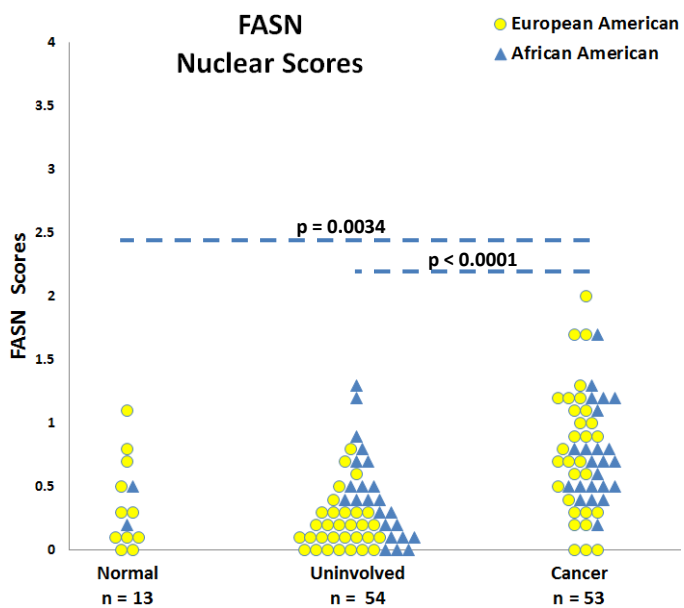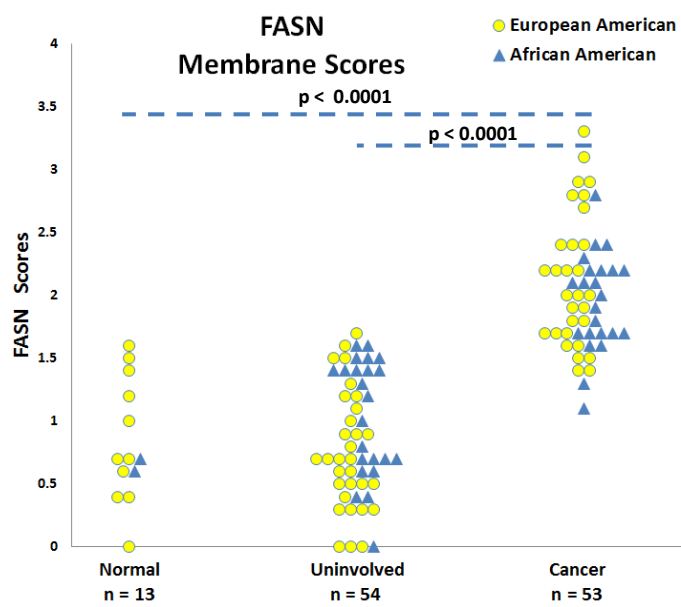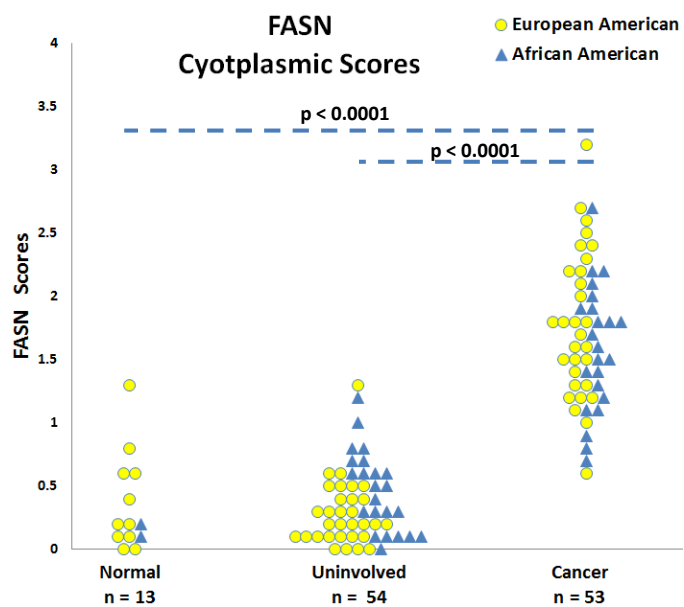
Legend

Figure IHC-5 Expression of FABP1 in PrCa. This figure demonstrates the range of expression of FABP1 in tumors broken down by Gleason scores (GS). These are the cases randomly chosen. In the next quarter we will target GS case of 6, 4+3, and 8-10 that are
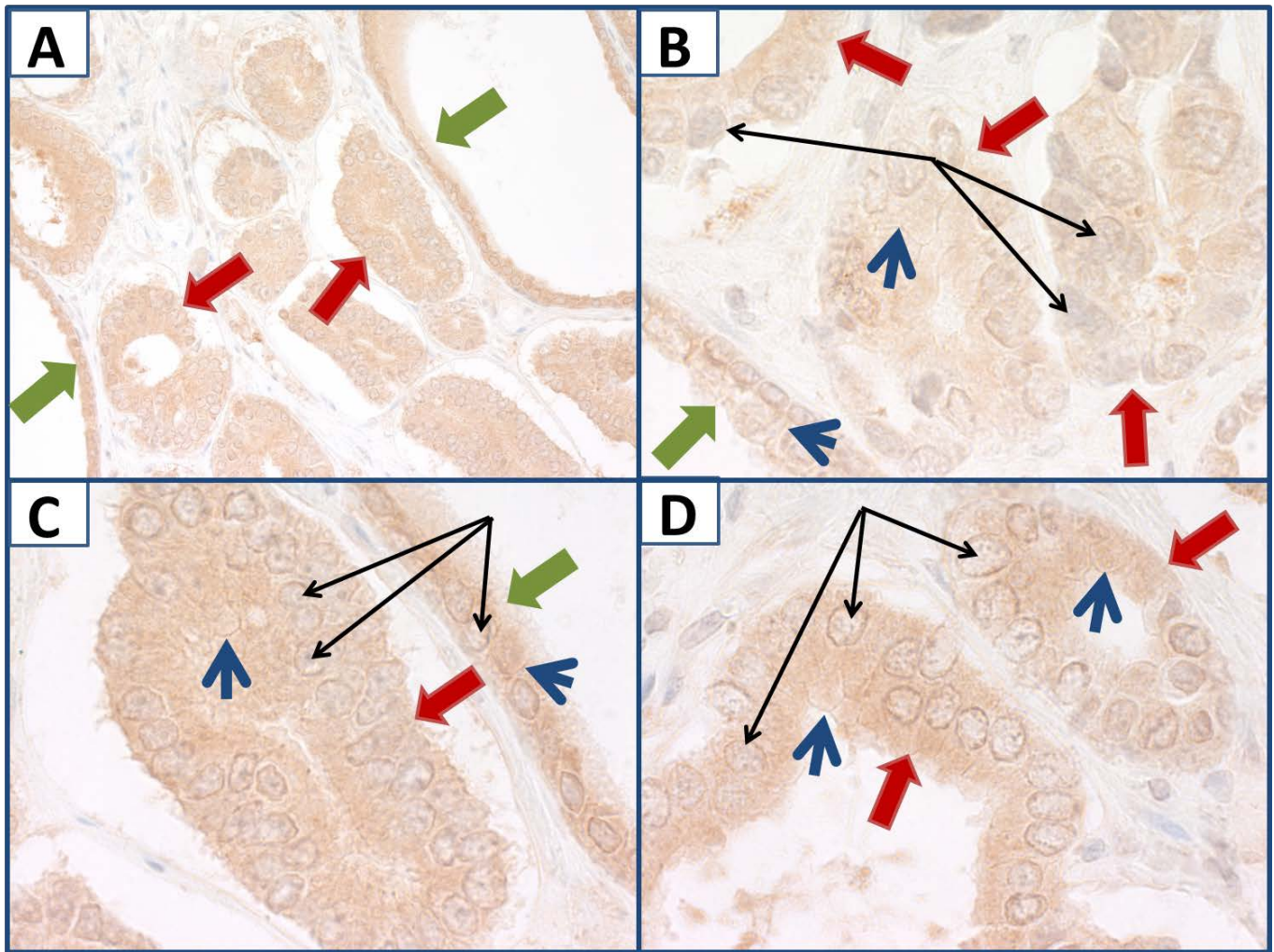
Legend:
Figure IHC-6 Expression of FABP1 in PrCa. Panel A (x200) demonstrates the intracellular expression of FABP-1 in an area of PrCa (red block arrows) adjacent to uninvolved prostate glands (green block arrows). At x630 magnification, in panels B, C and D, the luminal cells of uninvolved prostate glands (green block arrows) and PrCa (red block arrows) have membrane expression (blue arrows) while the thin black arrows point to nuclear staining of FABP-1 in PrCa. In this case the staining of the nuclei for FABP-1 is less than cytoplasmic staining.

There is consistent staining of the cytoplasm with FABP1 with increased staining of the cell membranes in both uninvolved luminal cells and PrCa cells. There is little differences in the expression of FABP1 between PrCa and uninvolved luminal cells; however, because of the large number of cases, the differences are statistically significant. Overall this pattern is consistent with the results summarized in Figure IHC-4.

FASN
Cyotplasmic Scores

FASN
Membrane Scores

FASN
Nuclear Scores

FASN
Perinuclear Scores
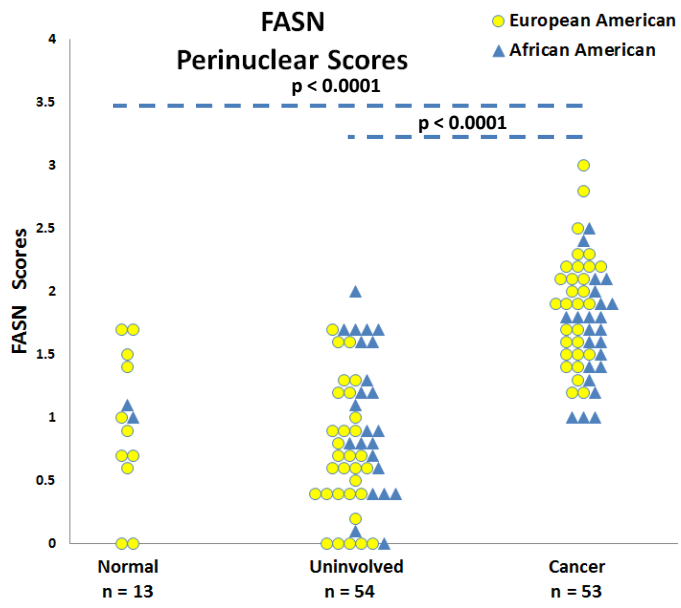
Legend

Figure IHC-7 Expression of FASN in the prostate. This figure demonstrates the differential expression of FASN in glands of "normal" prostate compared to "cancer" (PrCa) (p<0.0001). The normal prostate glands are from prostatectomies removed as part of radical cystectomies that were found on pathologic examination to not have PrCa. Similarly, uninvolved (normal appearing) prostate glands were compared with the matching PrCas as to the differential expression of FASN (both p<0.0001).

Of note, most of the higher values of FASN (cytoplasmic and membrane) were in the EA population in contrast to FABP5; however, this was not the case in the uninvolved prostate in which AAs predominated. A similar pattern was seen in perinuclear staining (p<0.0001). In nuclear staining the pattern also was similar for uninvolved (p<0.0001) but not in the normal prostate. Normal versus PrCa showed differential staining (p=0.0034).
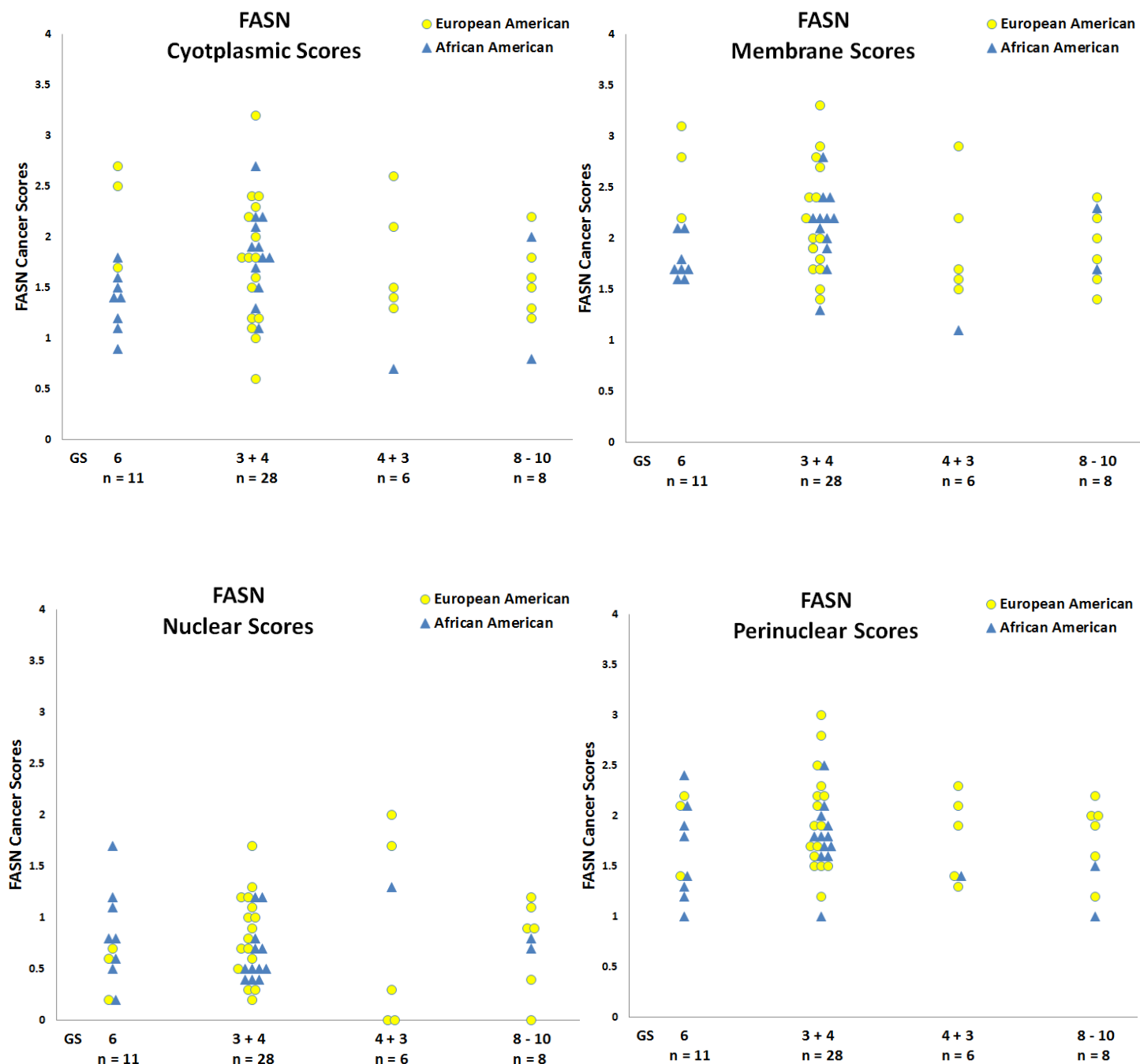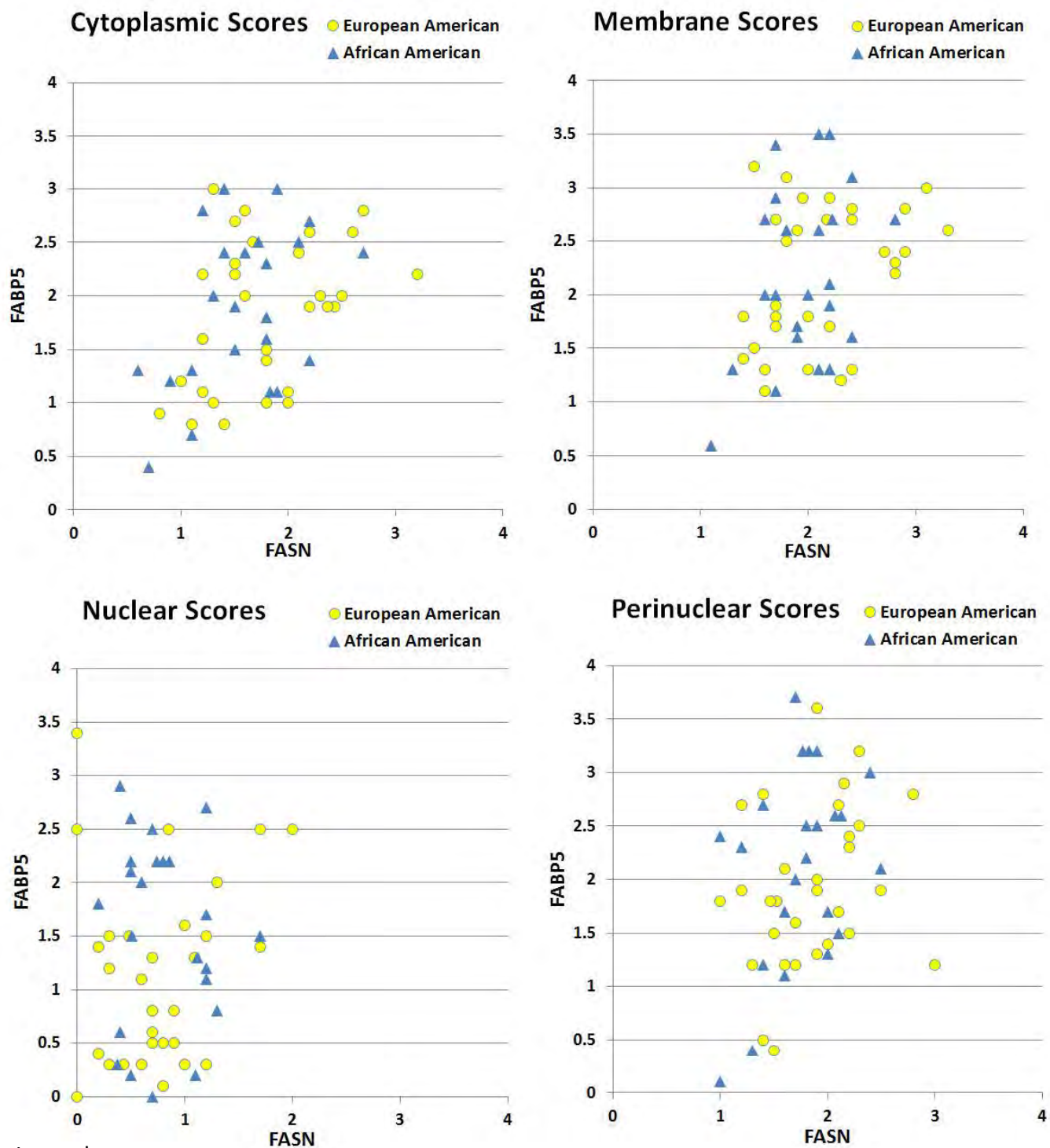
Legend

Figure IHC-8 Expression of FASN in PrCa. This figure demonstrates the expression of FASN in PrCa separated by Gleason scores (GS). These cases were randomly selected and results are expressed at cytoplasmic, membrane, nuclear and perinuclear intracellular patterns. Of note, we need more cases of Gleason scores 6, 4+3, and 8-10. Of importance, the higher values of FASN tend to occur in EA patients. In the next quarter we will add the needed cases and adjust the racial mix.
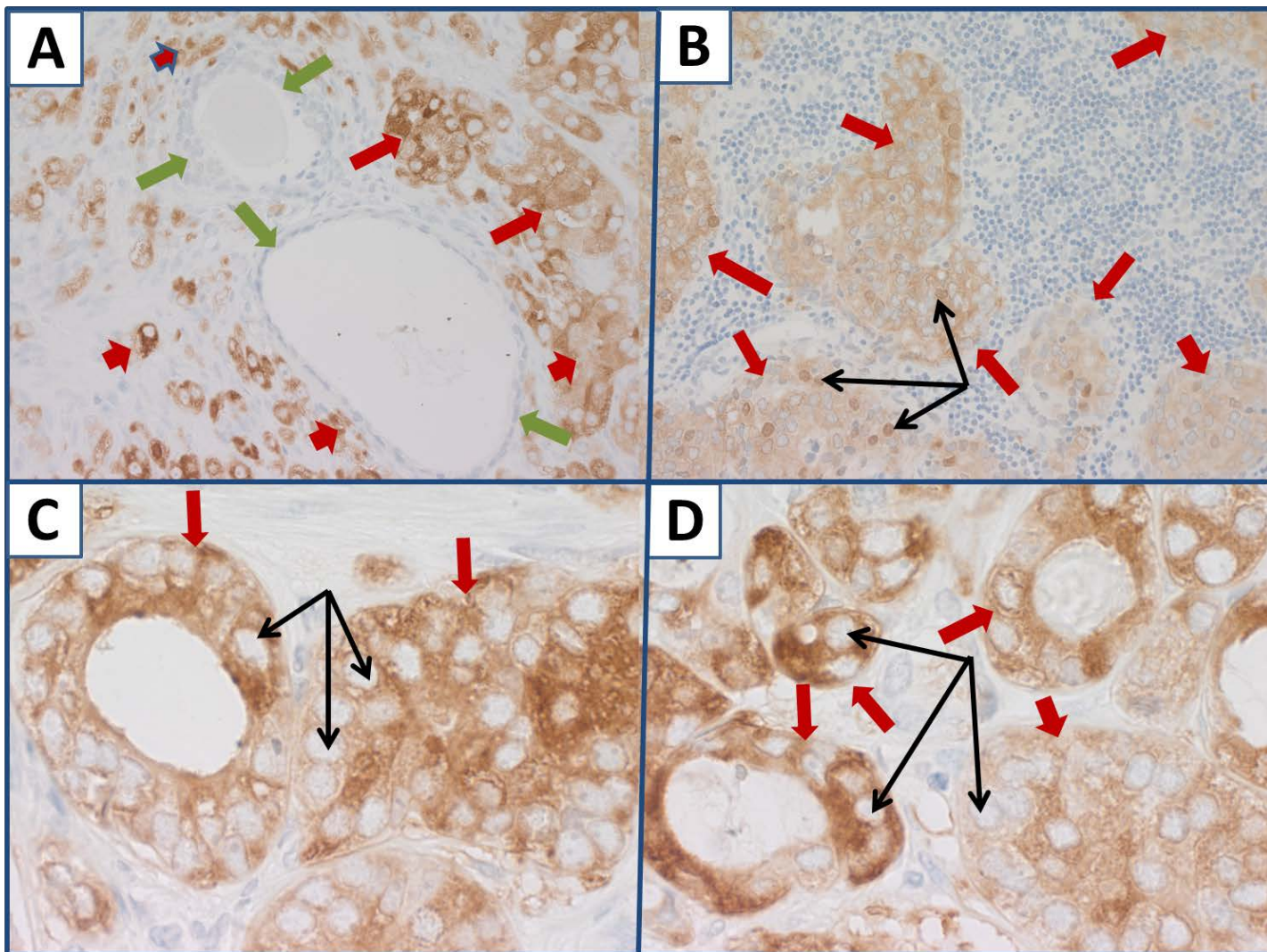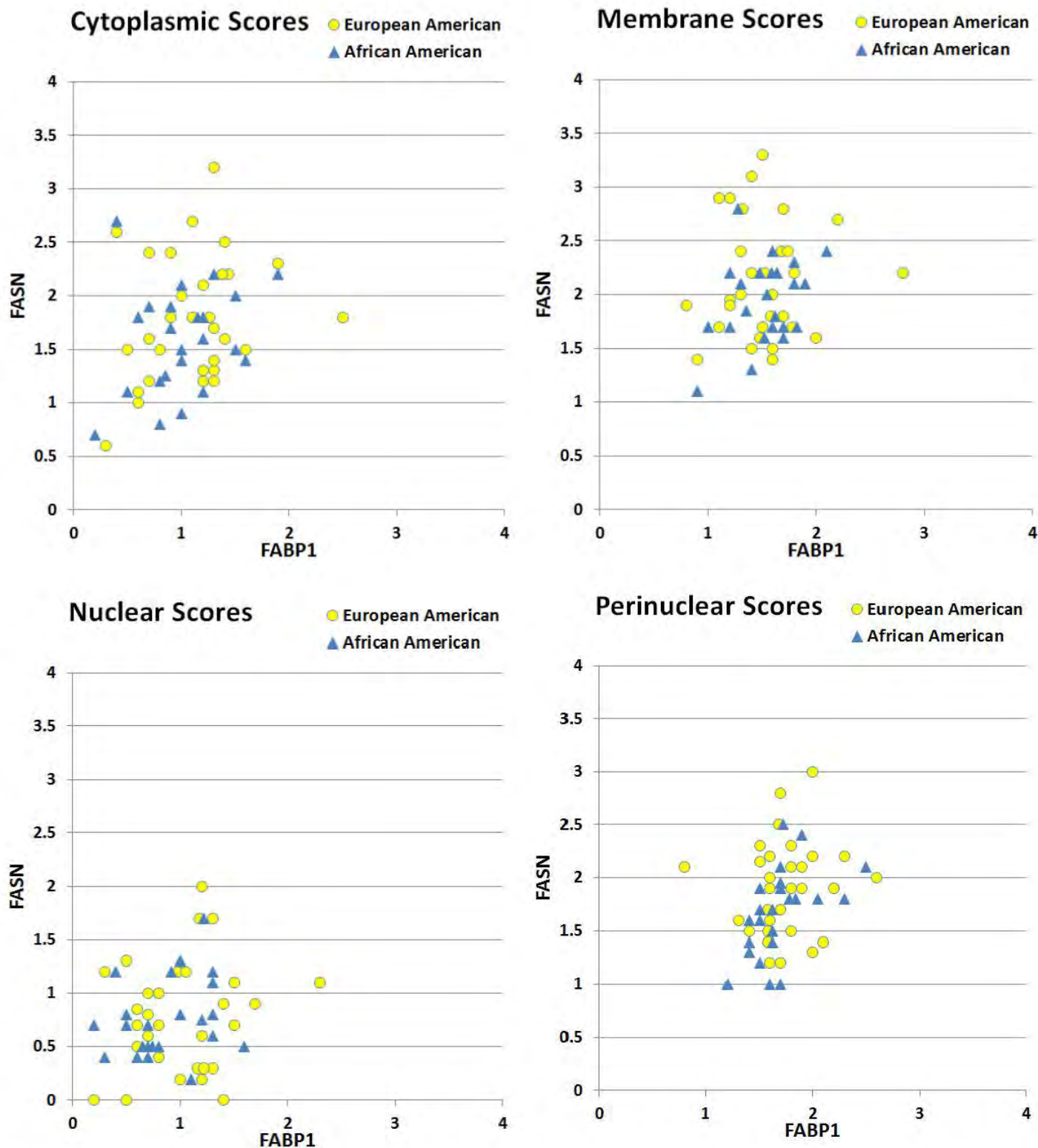
Legend
Figure IHC-9 Expression of FASN in the prostate. Panel A original magnification x200 demonstrates an area where high grade PrCa (red block arrows) surrounds uninvolved prostate ducts (green block arrows). Panel B original magnification x200 is an area in which foci of PrCa (red block arrows) are surrounded by lymphocytes which are not stained by FASN. In this high grade PrCa, the thin black arrows point to nuclei in which FASN is expressed. Panel C (x630) demonstrates high grade prostate cancer (red block arrows) with most nuclei that have no FASN staining as does Panel D (x630). However, some nuclei of both Panels C and D also contain nuclei which are stained with FASN.
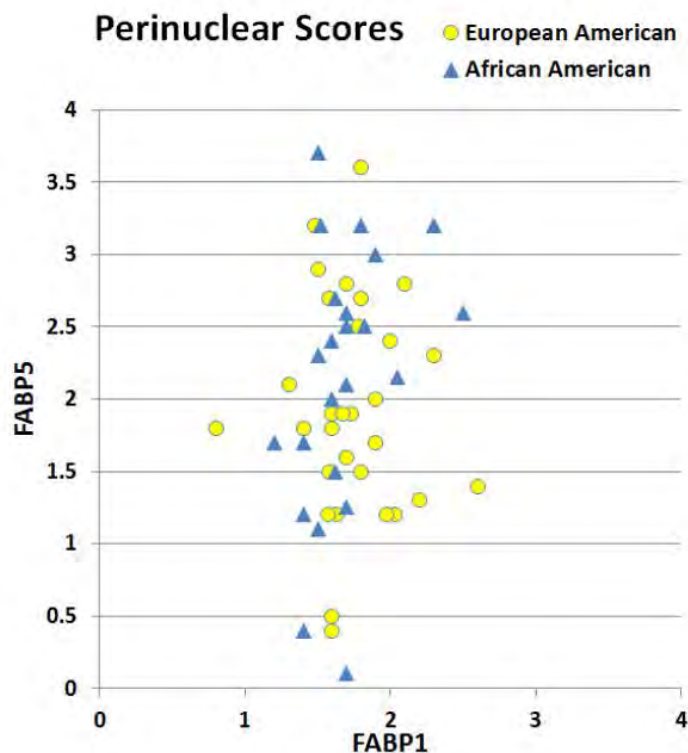
Legend

Figure IHC-10 Correlation of FABP5 expression with the expression of FASN in PrCa. This figure was prepared to test if the same changes observed at the mRNA level in which, for some case of PrCa, the mRNA of FABP5 and FASN were oppositely expressed (i. e., ↑ FASN, ↓ FABP5, or ↓FASN, ↑ FABP5) were present at at the protein level. This pattern was not observed for any of the intracellular components. What is observed is that higher FABP5 scores tend to be enriched in AAs and higher FASN scores are enriched in EAs (Table IHC-1). This is especially apparent for nuclear and perinuclear expression.

Cytoplasmic Scores ○ European American ▲ African American

Membrane Scores ○ European American ▲ African American

Nuclear Scores ○ European American ▲ African American

Perinuclear Scores ○ European American ▲ African American

Legend

Figure IHC-11 Correlation of FASN with FABP1 in PrCa. As with Figure IHC-10, no apparent pattern of opposite expression between FASN and FABP1 was noted. A pattern of increased expression of FASN in EAs is apparent at the cytoplasmic, membrane, nuclear and perinuclear areas of the cell.
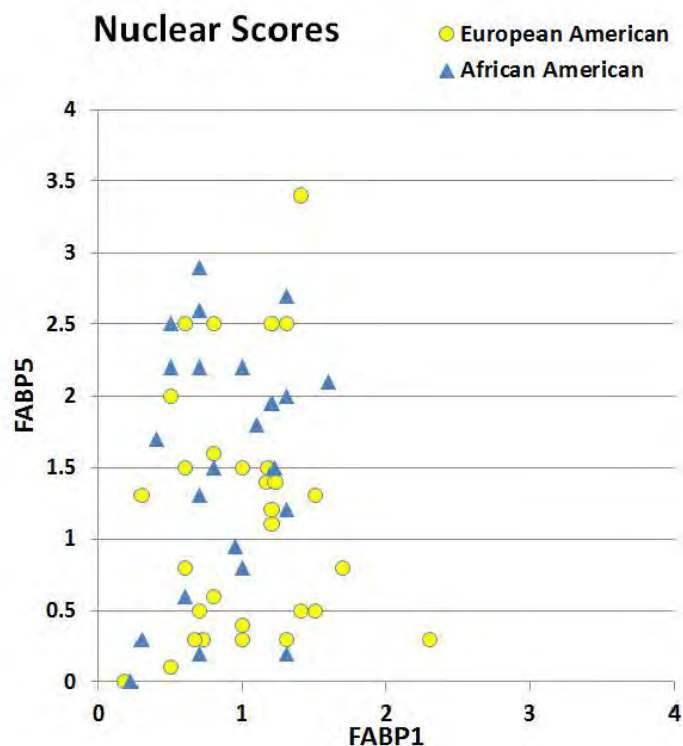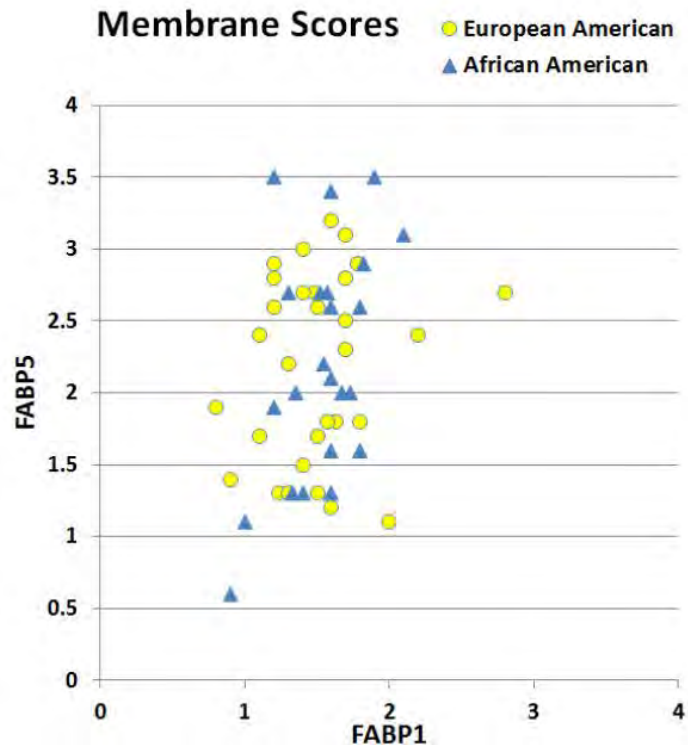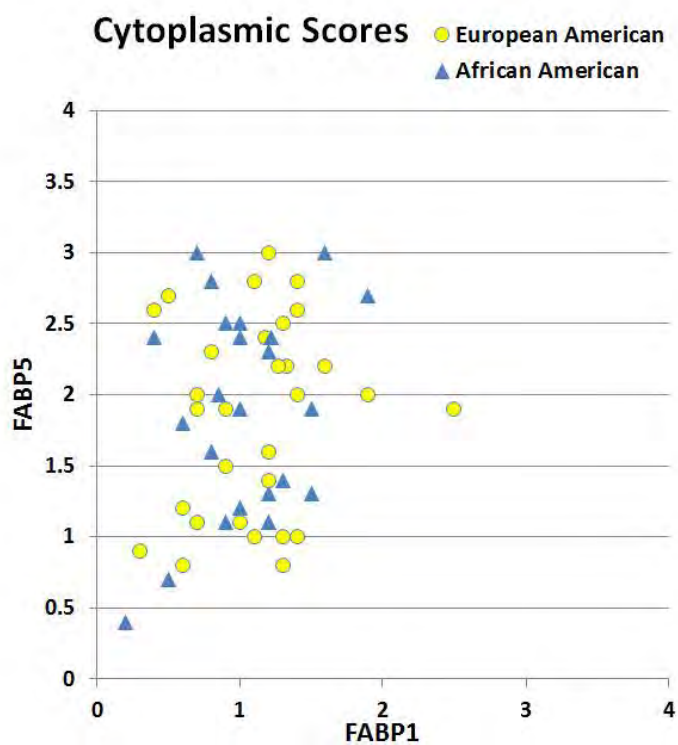
Legend

Figure IHC-12 Correlation of FABP5 with FABP1 in PrCa. Again, this does not appear to be an inverse correlation between the expressions of FABP5 with FABP1. Of note compared with FABP1, there is an relative increase in FABP5 expression in AAs at the cytoplasmic, membrane, nuclear, and perinuclear areas of malignant cells. This is especially apparent in the nuclear and perinuclear areas of the cells.

Table IHC-1 Comparison of FABP5 versus FASN Phenotypic Expression

Marker and Intracellular Component		AAs	EAs
FABP5	Cutoff	% ≥ Cutoff	% ≥ Cutoff
Cytoplasmic	2.5	26	23
Membrane	2.5	43	42
Nuclear	2.0	35	19
Perinuclear	2.5	35	26
FASN			
Cytoplasmic	2.0	17	35
Membrane	2.5	4	23
Nuclear	1.0	34	29
Perinuclear	2.0	26	42

FIGURE MS-1
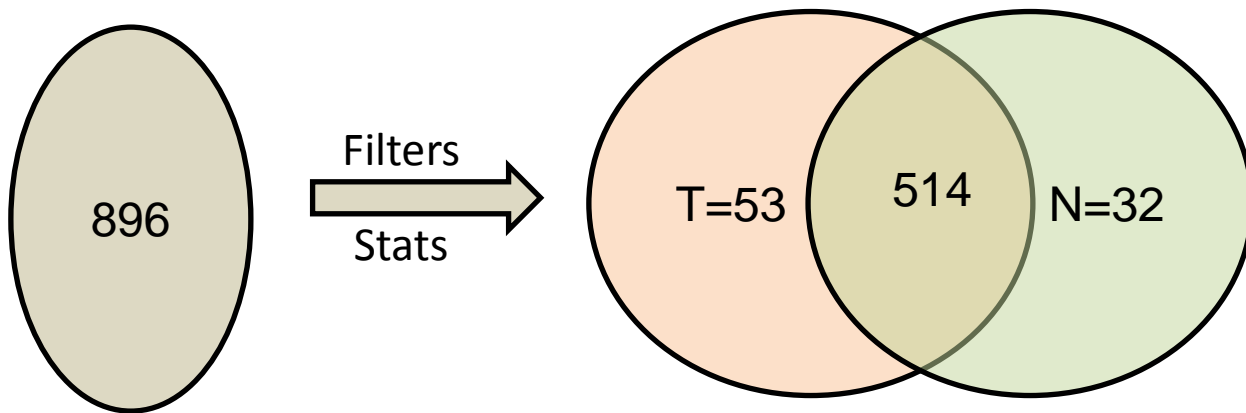
Legend
Figure MS-1
After filtering the data ~900 proteins were identified with <0.1% FDR. Of those, 514 proteins were found to be identified in >30% of patient specimens for each arm. We have found that at least 30% of samples per statistical arm must have quantifiable peptides in order to obtain robust analysis (we call this a commonality filter). However, for specific proteins of interest we do go back and pull out the data to identify potential proteins of interest.

FIGURE MS-2
Systems Analysis
(GO Associated Localizations)

Cellular Component GO Term Annotation Comparison

Legend
Figure MS-2
Systems analysis demonstrating the cellular components from
which the modulated proteins of prostate cancer are associated.

FIGURE MS-3
Systems Analysis
(GO Associated Processes)

Biological Process GO Term Annotation Comparison
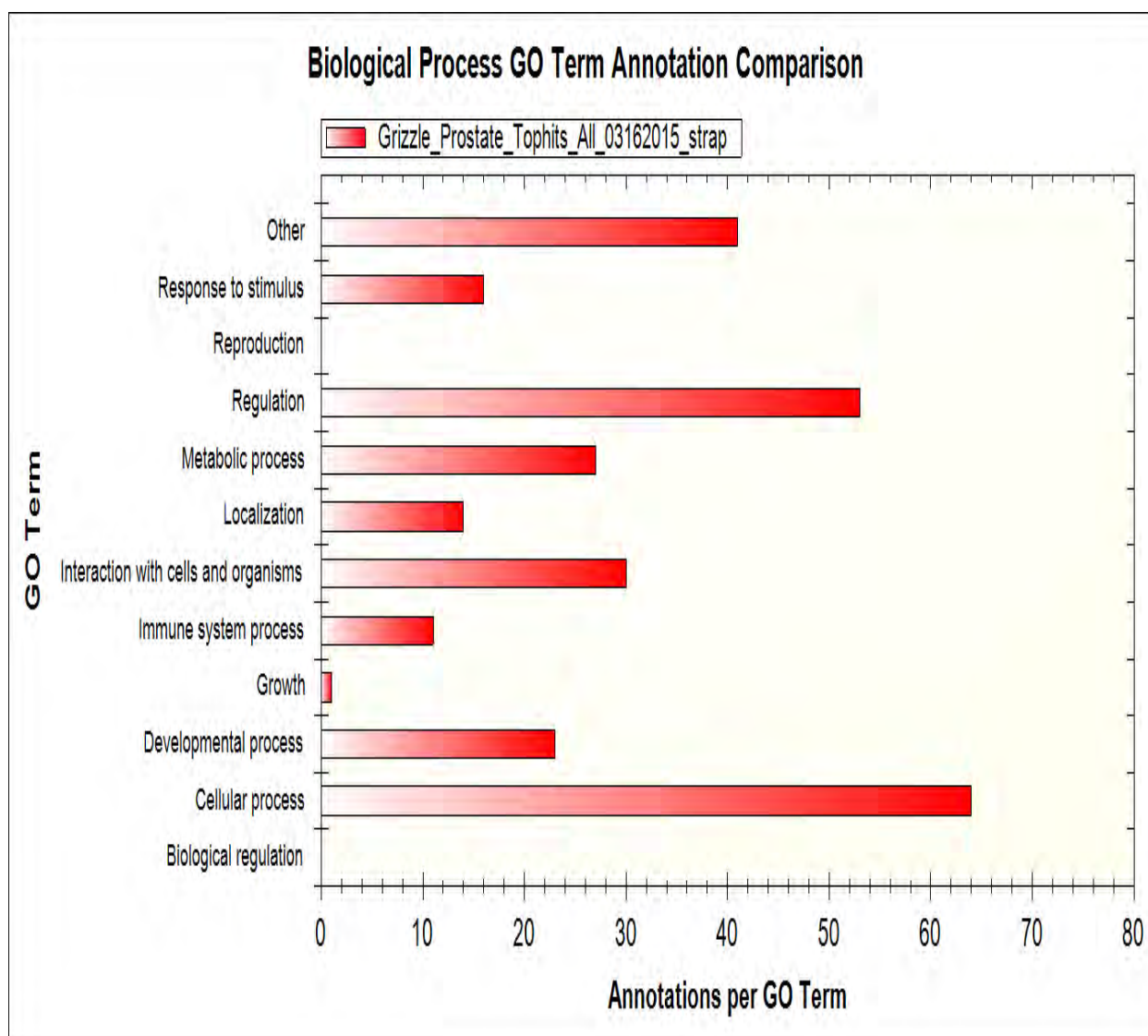
Legend Figure MS-3
Systems analysis demonstrating the biological process associated with the modulated proteins of PrCa.

FIGURE MS-4

Protein Abundance Changed in Prostate Biopsies
(Tumor vs. Matched Uninvolved)
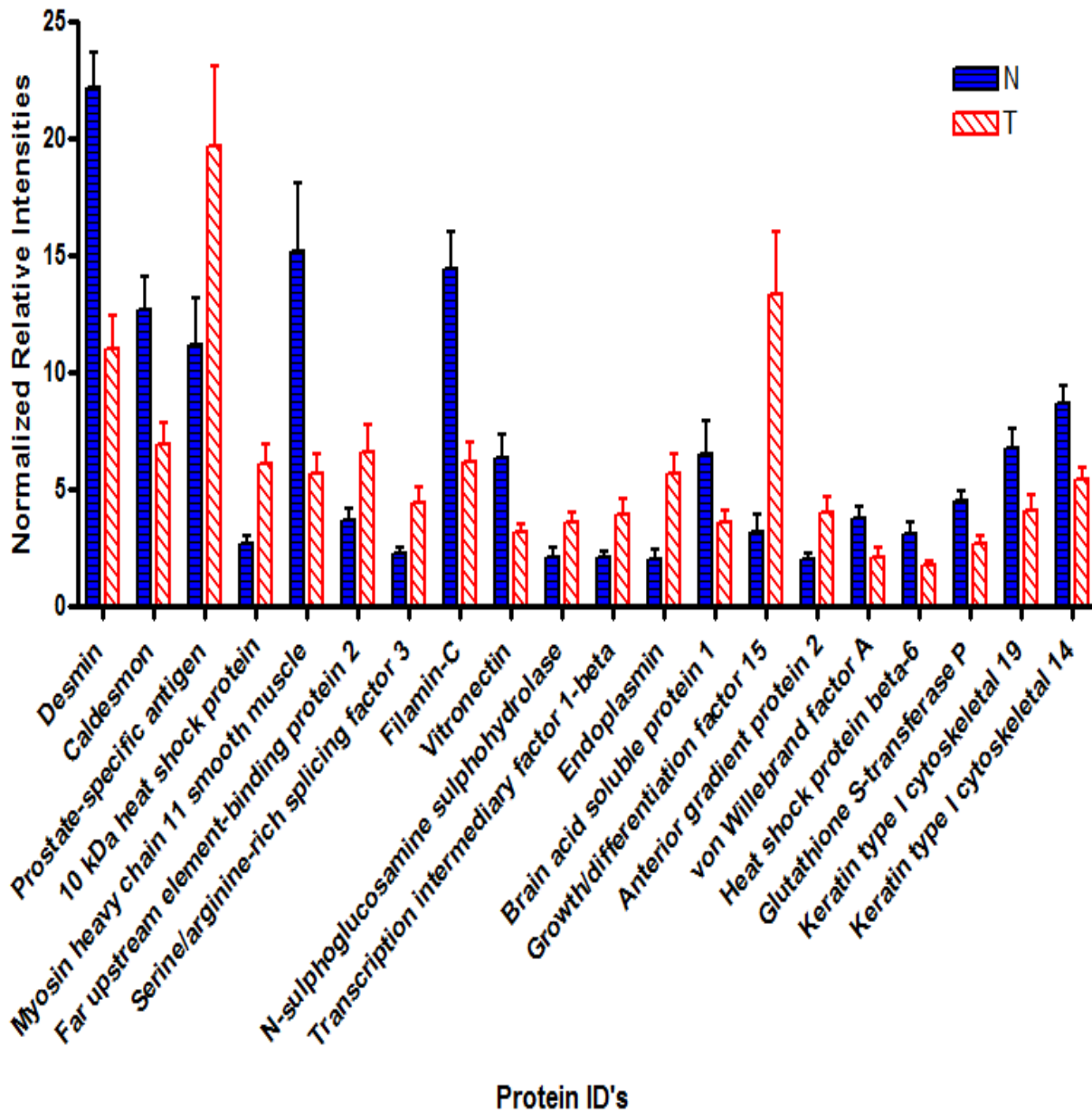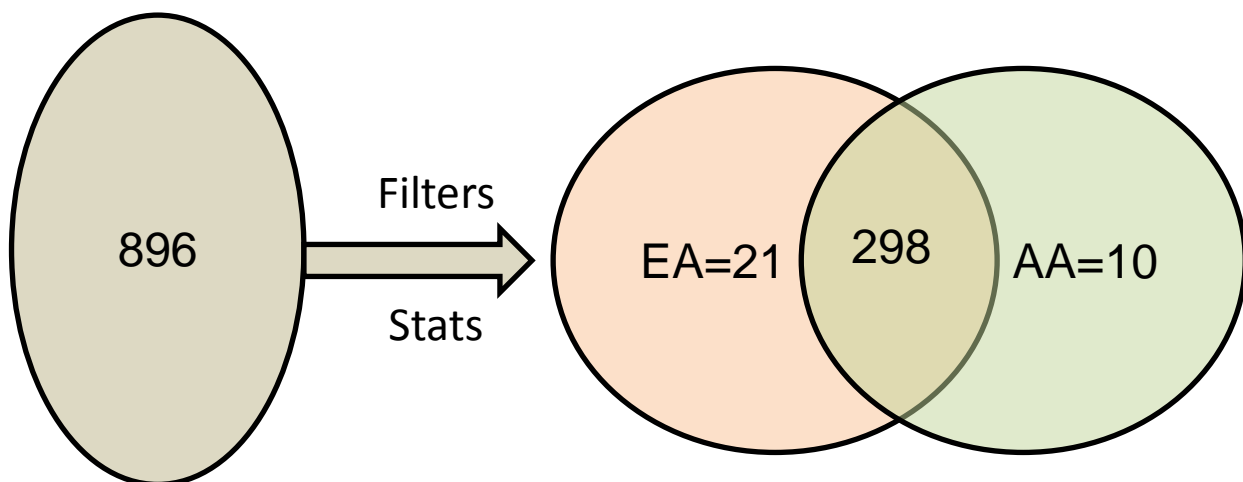
Legend

FIGURE MS-5

Legend
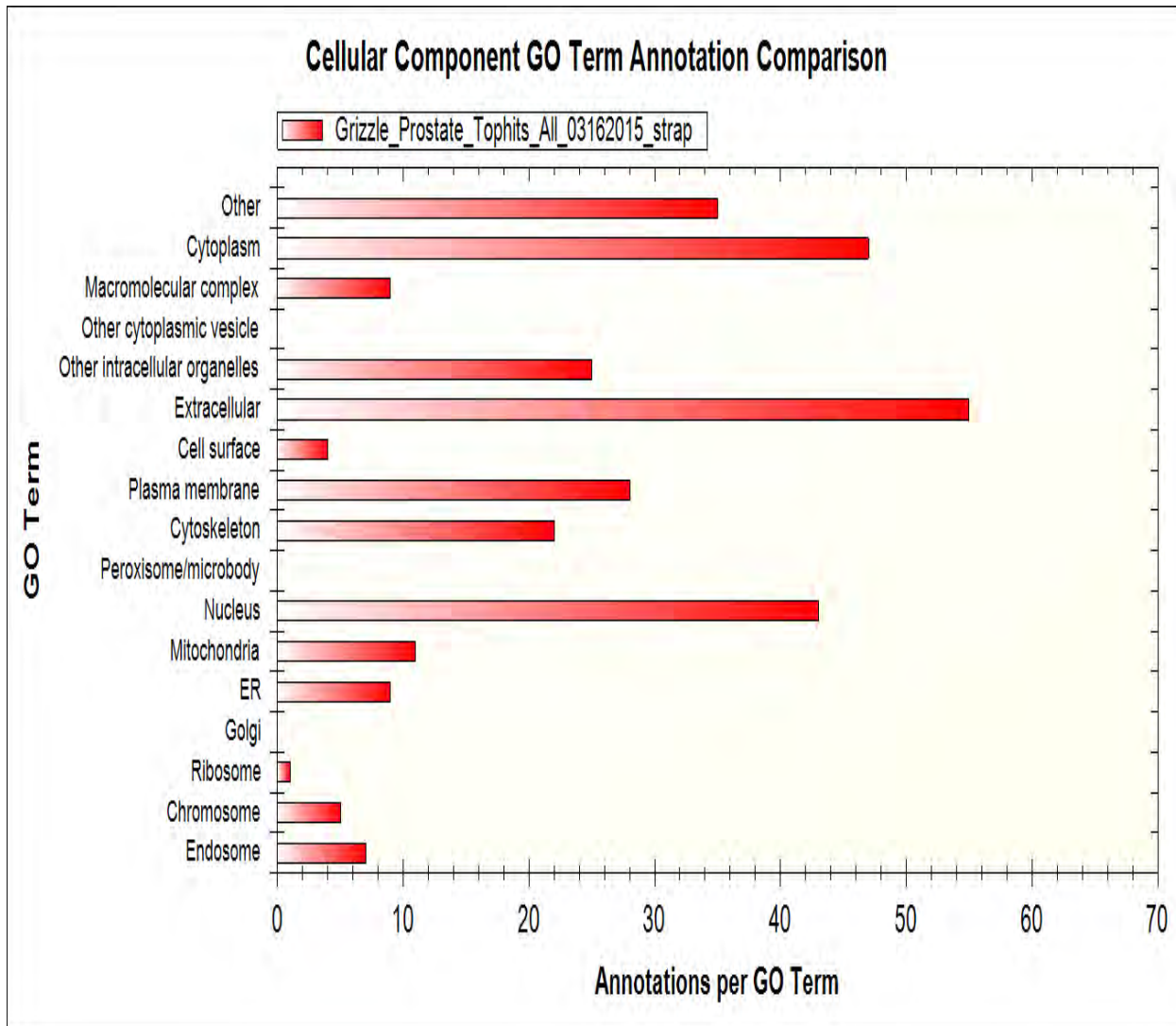**Figure MS-5**
After filtering the data ~900 proteins were identified with <0.1% FDR. Of those, 298 proteins were found to be identified in >50% of patient specimens for each arm. We have found that at least 50% of samples per statistical arm must have quantifiable peptides when one arm is limited as is the case here. The AA arm was limited to 8 patients and therefore we had to go with a limit of 4 patients with

quantifiable data in order to obtain robust analysis (we call this a commonality filter).

TABLE MS-1
10 Proteins Increased in Tumor Tissues of AAs vs EAs

Protein Name	Comments	Accession #	Network Name	SAM	Ttest	Fold (T-AA/EA)
Keratin, type II cytoskeletal 5	Attaches to K14 to form intermediate filaments and hence cell connections anchored to desmosomes; primarily focused in stratified epithelium	P13647	Keratin 5	0.48	0.029	1.5
Alpha-actinin-4	Cancer cell motility Met?	O43707	Alpha-actinin	0.62	0.024	1.6
Ubiquitin-like modifier-activating enzyme 1	Involved in conjugation to ubiquitin; targeted in cancer	P22314	UBA1	0.52	0.030	1.7
Alpha-1-antichymotrypsin	Less in prostate cancer; high in pancreatic cancer; lower in cancer than uninvolved; higher in more advanced cancers	P01011	SERPINA3 (act)	0.47	0.040	1.7
40S ribosomal protein S17		POCW22	RPS17	0.58	0.046	1.7
Zinc-alpha-2-glycoprotein	Stimulates lipolysis; regulated by glucocorticoids; tumor biomarker; cachexia of cancer	P25311	AZGP1	0.54	0.023	1.8
Galectin-3-binding protein	Cancer associated protein affects motility; metastasis (?)	Q08380	90K	0.60	0.022	1.8
Malate dehydrogenase 2		Q6FHZ0	MDH2	0.58	0.035	1.9
Prostate-specific antigen		P07288	Kallikrein 3 (PSA)	0.61	0.008	2.2
Complement component 4A		B2RUT6	C4	0.63	0.058	2.7

Of the 10 proteins increased in prostate cancer from AA patients compared to EA patients, 3 of the proteins may be involved in the aggressiveness of prostate cancer in AAs. We found this encouraging and plan to increase the power of the study by macrodissecting and analyzing additional cases by mass spectrometry.

TABLE MS-2
21 Proteins Decreased in Tumor

Protein Name	Accession #	Network Name	SAM	Ttest	Fold(T-AA/C)
Glutaredoxin-1	P35754	Glutaredoxin	0.79	0.013	-2.3
Tubulin beta-6 chain	Q9BUF5	Tubulin beta	0.87	0.003	-2.2
Hematological and neurological expressed 1	Q9H910	HN1L	0.60	0.016	-2.0
Cadherin-1	P12830	CDH1	0.57	0.032	-1.9
Vitronectin	P04004	Vitronectin	0.70	0.006	-1.9
Beta-2-microglobulin	P61769	Beta-2-microglobulin	0.58	0.040	-1.8
40S ribosomal protein S19	P39019	RPS19	0.60	0.010	-1.8
Protein canopy homolog 2	Q9Y2B0	MSAP	0.58	0.040	-1.8
Hemoglobin subunit delta	P02042	Adult hemoglobin	0.63	0.008	-1.8
N-sulphoglucosamine sulphohydrolase	P51688	SPHM	0.70	0.007	-1.8
Brain acid soluble protein 1	P80723	BASP1	0.49	0.039	-1.8
Ubiquitin-conjugating enzyme E2	Q15819	MMS2	0.67	0.020	-1.7
Cathepsin Z	Q9UBR2	Cathepsin Z	0.69	0.016	-1.7
Laminin subunit alpha-5	O15230	LAMA5	0.55	0.020	-1.7
Fibrillin-1	P35555	Fibrillin	0.43	0.040	-1.7
Laminin subunit gamma-1	P11047	LAMG1	0.59	0.011	-1.7
Myosin light chain kinase	Q15746	MLCK	0.59	0.012	-1.6
Beta-globin	C8C504	HBB	0.56	0.014	-1.6
Protein disulfide-isomerase	P07237	P4HB	0.51	0.048	-1.6
Apolipoprotein A-I	P02647	APOA1	0.44	0.050	-1.6
ETHE1	O95571	HSCO	0.49	0.042	-1.5

FIGURE MS-6

FFPE Prostate Tissue Biomarkers Compared Between AAs vs EAs (PrCa – p<0.05 for AAs vs EAs only)
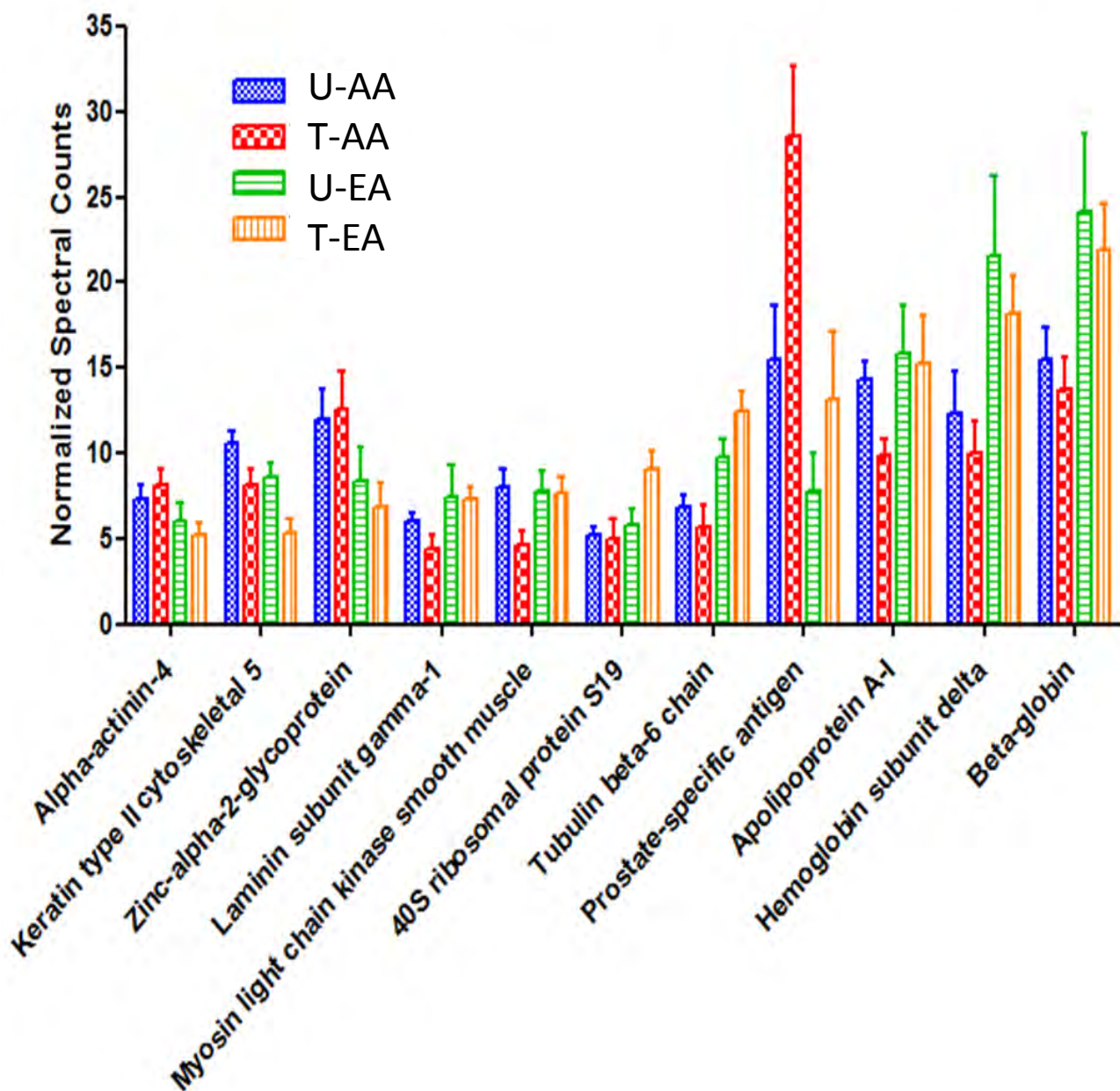
Legend
Figure MS-6

All values are indicated for those proteins that are significantly changed in PrCa tissues for AAs vs EAs.

FIGURE MS-7

FFPE Prostate Tissue Biomarkers Compared Between AA vs. C
(PCa - p<0.05 for AAvC only)

Legend: U-AA, T-AA, U-C, T-C

Legend
Figure MS-7
All values are indicated for those proteins that are significantly changed in PrCa tissues for AAs vs EAs.

FIGURE MS-8

Fibrillin-1 Levels in FFPE Prostate Tissues Compared Between AAs vs EAs
(PrCa – p<0.05 for AAs vs EAs)
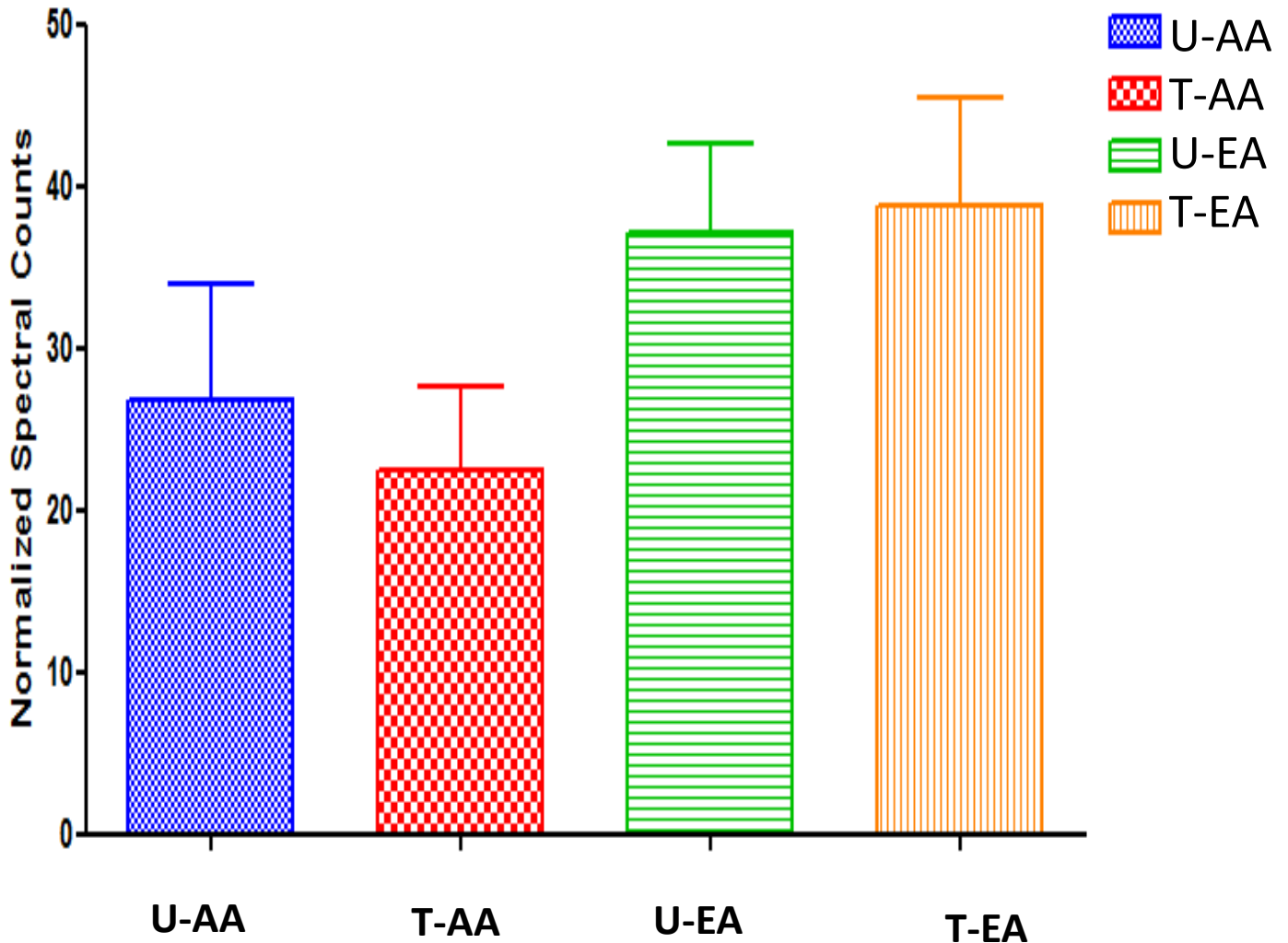
Legend
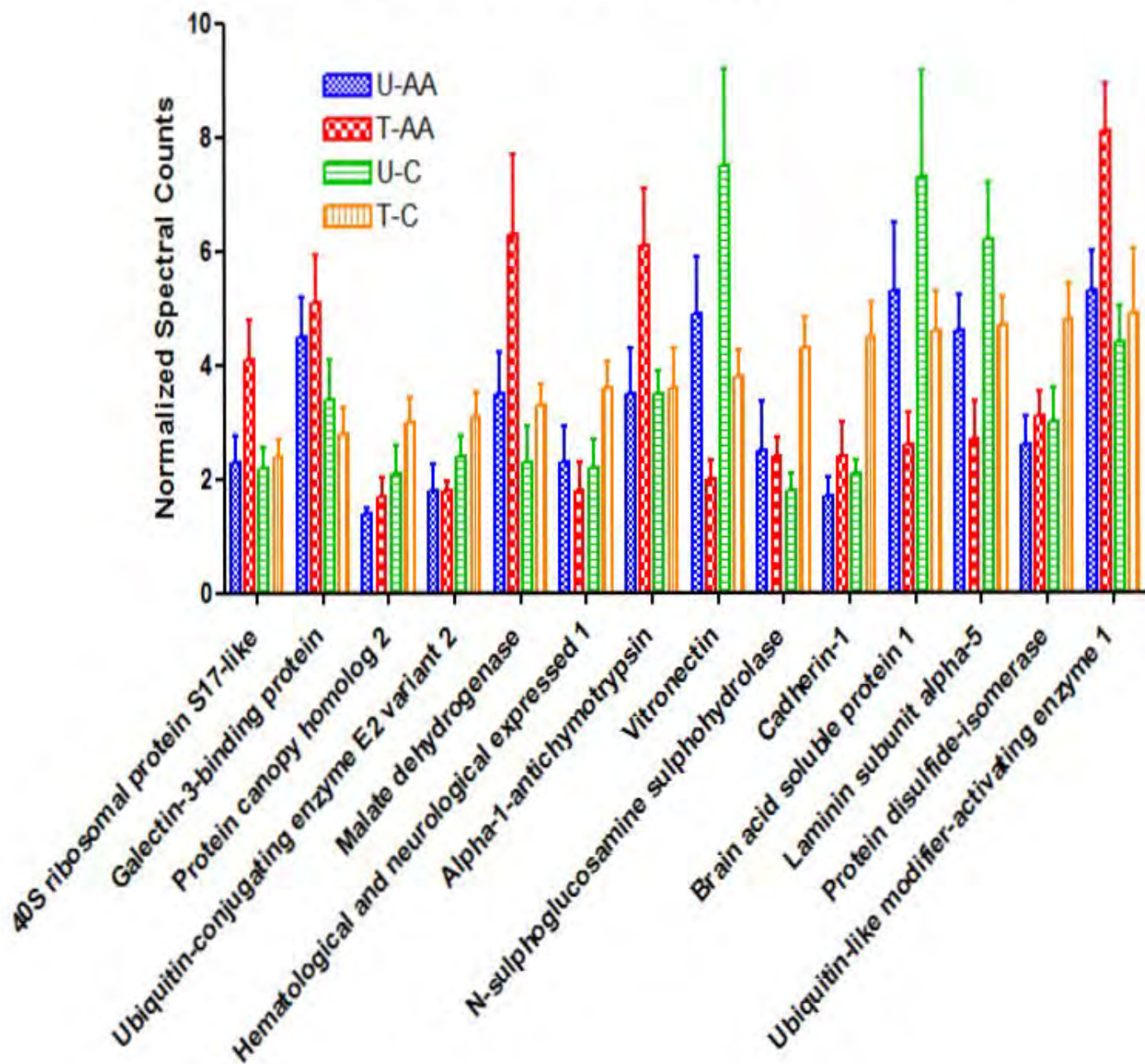**Figure MS-8**
This is just one example that was worth highlighting where all values are indicated for those proteins that are significantly changed in PrCa tissues for AAs vs EAs.

FIGURE MS-9
STRAP GO Analysis for Significantly Changed Proteins (PrCa - AAs vs EAs)
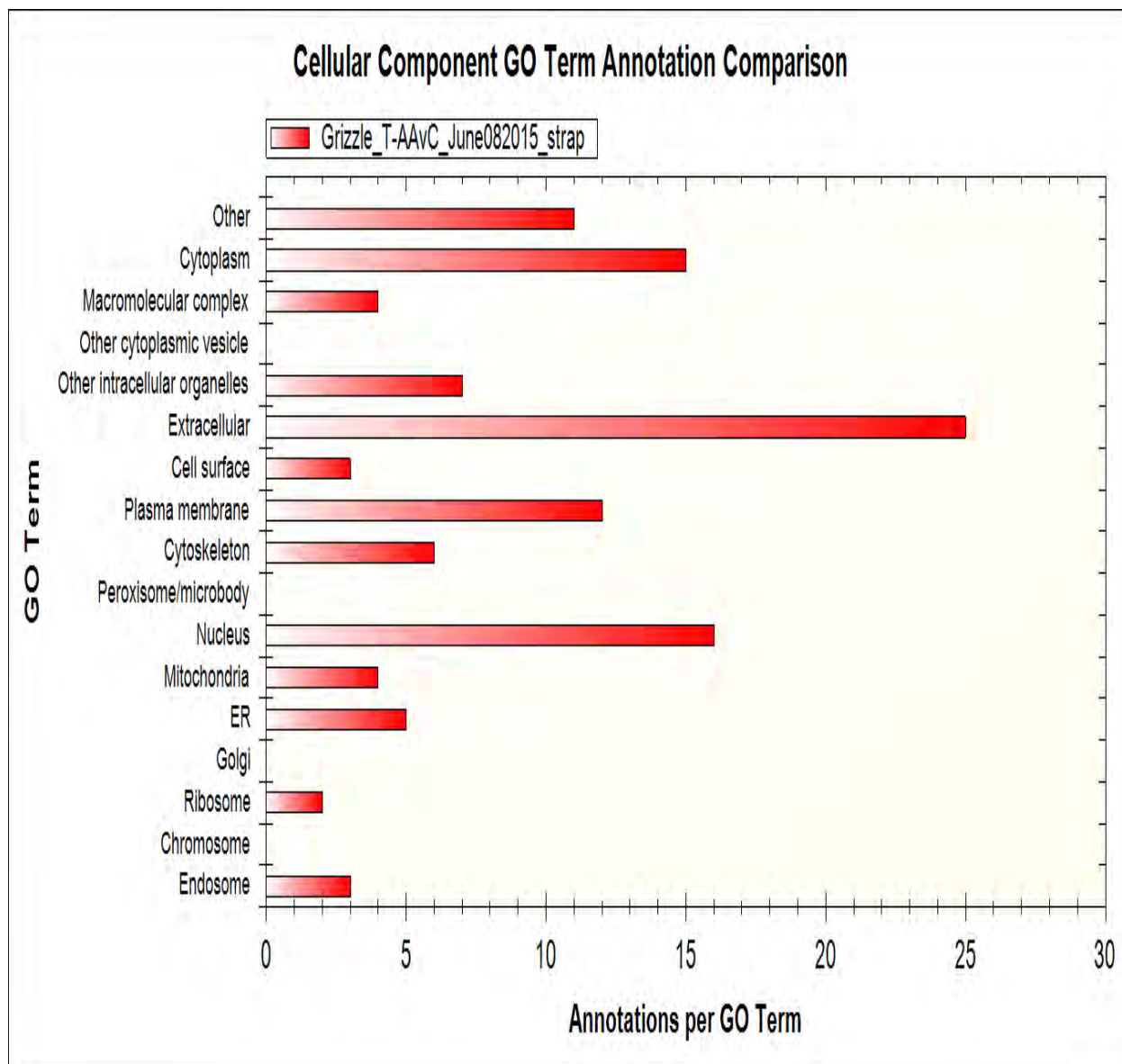
Legend
Figure MS-9
Systems analysis demonstrating the cellular components of modulated proteins of PrCa between AAs vs EAs.

FIGURE MS-10
STRAP GO Analysis for
Significantly Changed Proteins
(PrCa - AAs vs EAs)
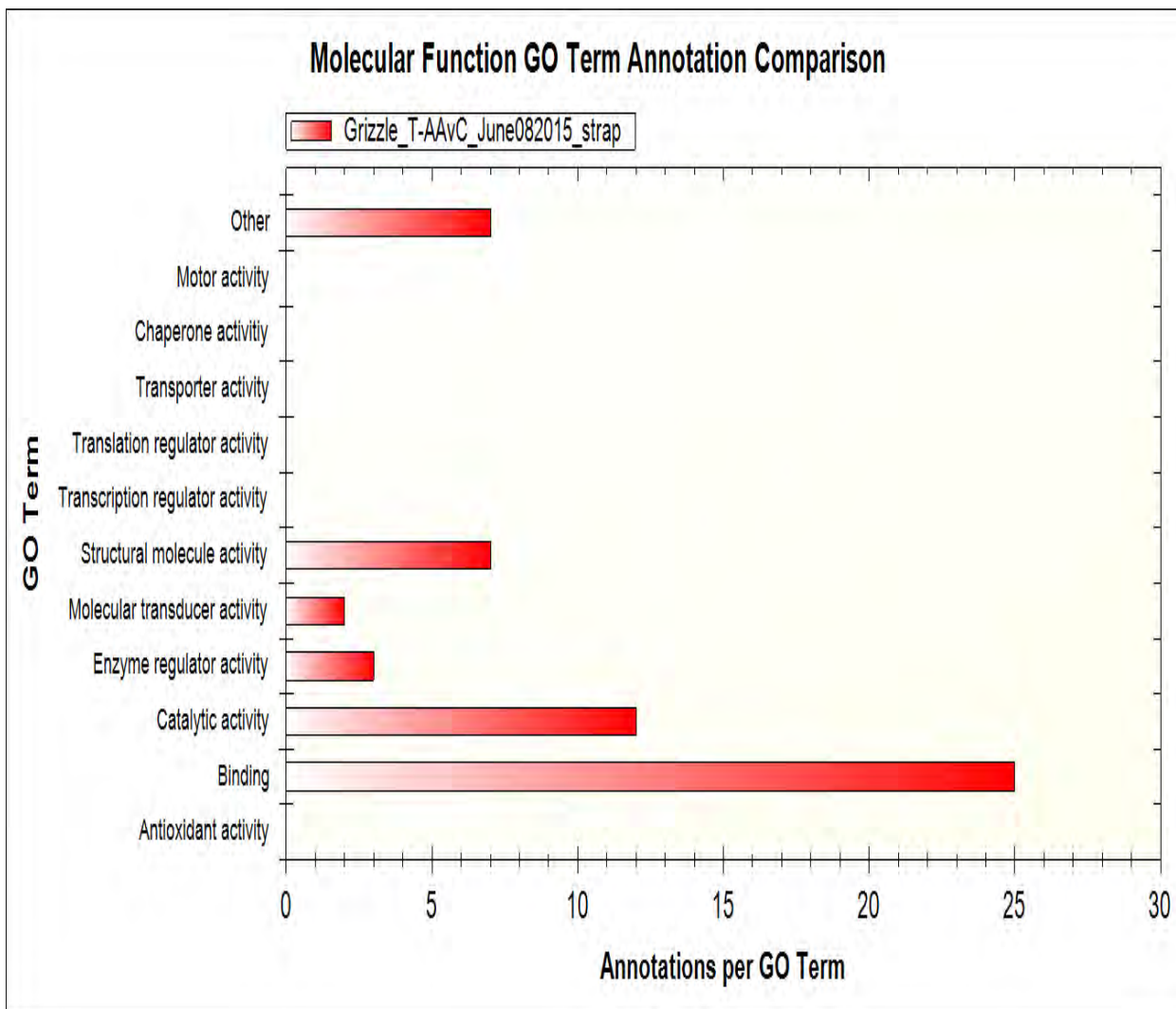
Legend
Figure MS-10
Systems analysis demonstrating the molecular function of
modulated proteins of PrCa between AAs vs EAs.

FIGURE MS-11
STRAP GO Analysis for
Significantly Changed Proteins
(PrCa - AAs vs EAs)
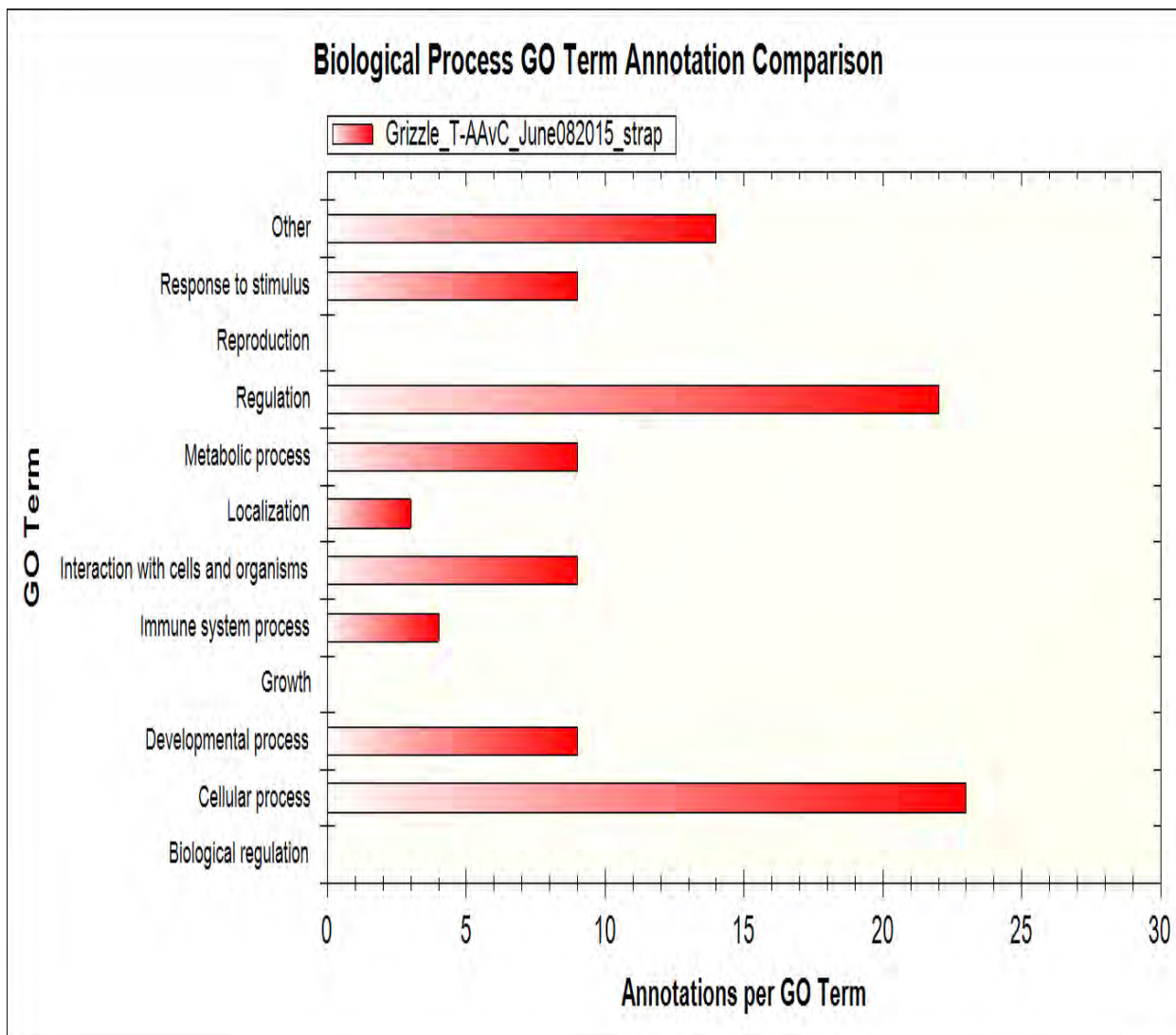
Systems analysis demonstrating the biological process of modulated proteins of PrCa between AAs vs EAs.

FIGURE MS-12
Network 1. SERPINA3 (ACT), Oncostatin M, BMP7, IL-6, Thrombopoietin

Legend Figure
MS-12

<u>Top Network with 19 protein hits:</u>
Regulation of…..
- ➢ Response to stress
- ➢ Immune response
- ➢ Defense response
- ➢ Response to stimulus
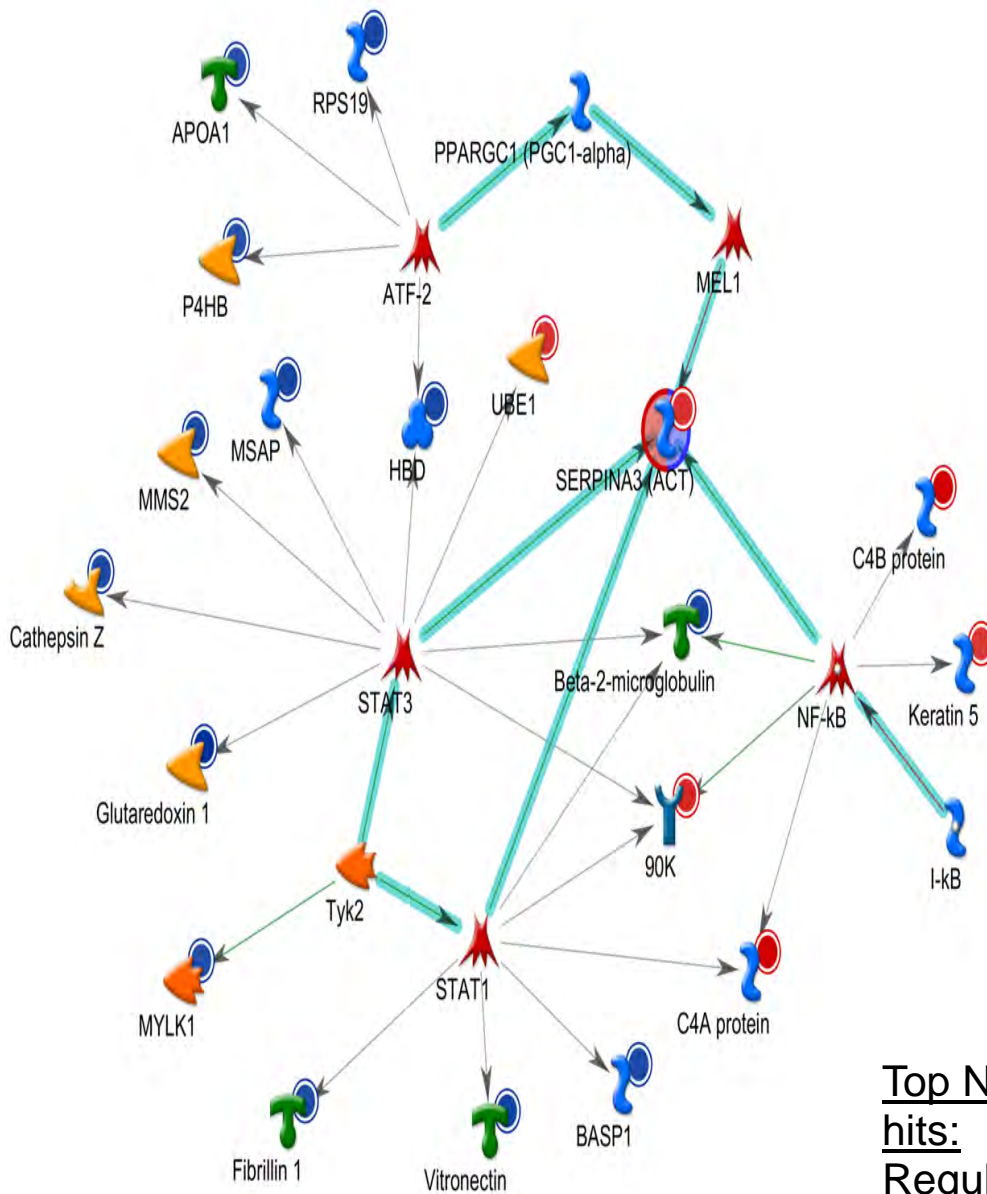
This is an example of a system analysis for SERPINA3.
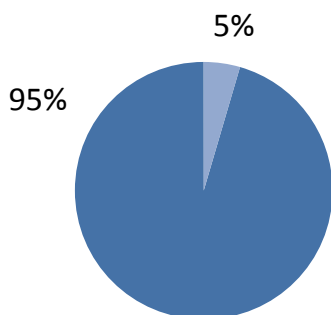
| Biopsy Diagnosis: High Grade PrCa N = 22 | Biopsy Diagnosis : Low Grade PrCa N = 18 | Biopsy Diagnosis: No PrCa N = 43 |
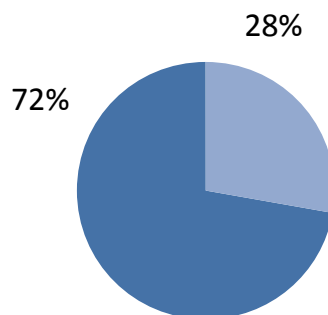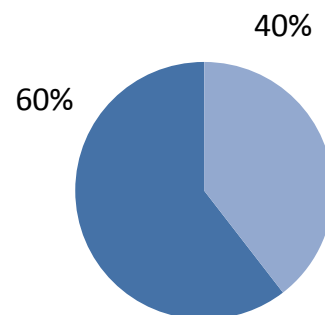
West African Ancestry 1.0 to 0.75   West African Ancestry 0.74 to 0.50

High grade PrCa (Gl sum 7 or more)

Low grade PrCa (Gl sum 6)

48

47

FABP5 - AA

FASN - AA
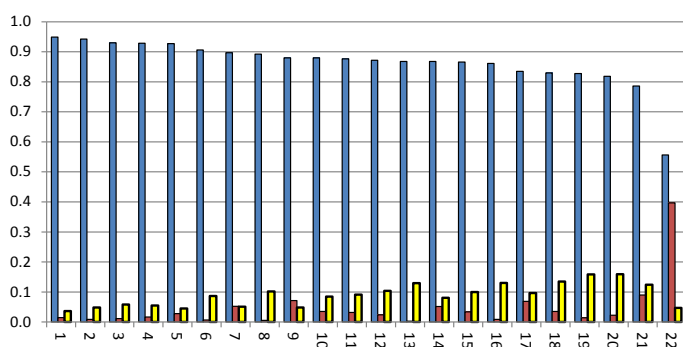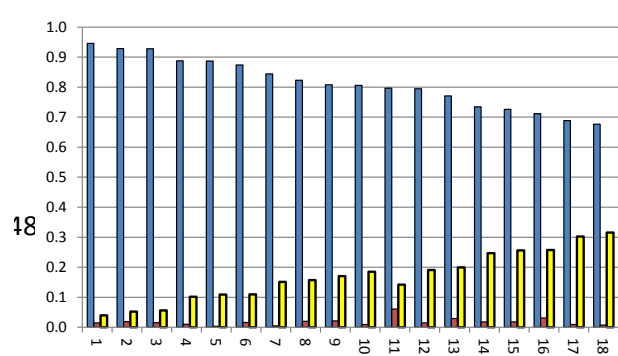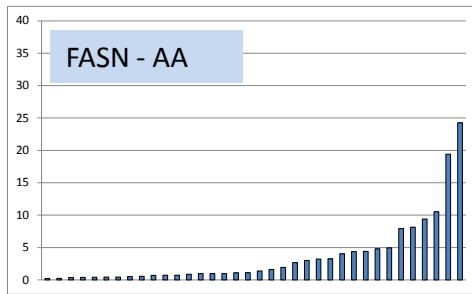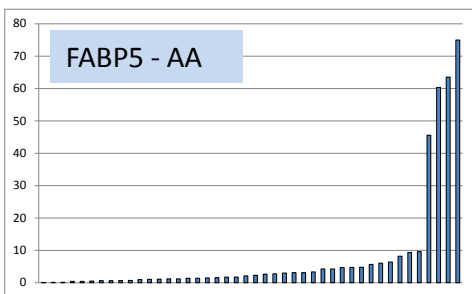
GE Figure 1: Outlier gene expression patterns of FABP5 and FASN in PrCa biopsies from AA and EA subjects

In our studies of prostate biopsies from our prostate biopsy study subjects, FABP5 and FASN mRNA expression showed patterns consistent with "outlier" PrCa subtypes. Further analysis of PrCa gene expression data available through Oncomine showed similar outlier patterns for FABP5 and FASN in at least four other independent studies. In our study subjects, the subgroup of cancers showing FABP5 super over-expression were predominant of AA origin, while the cancers showing FASN super over-expression were predominantly from EAs.
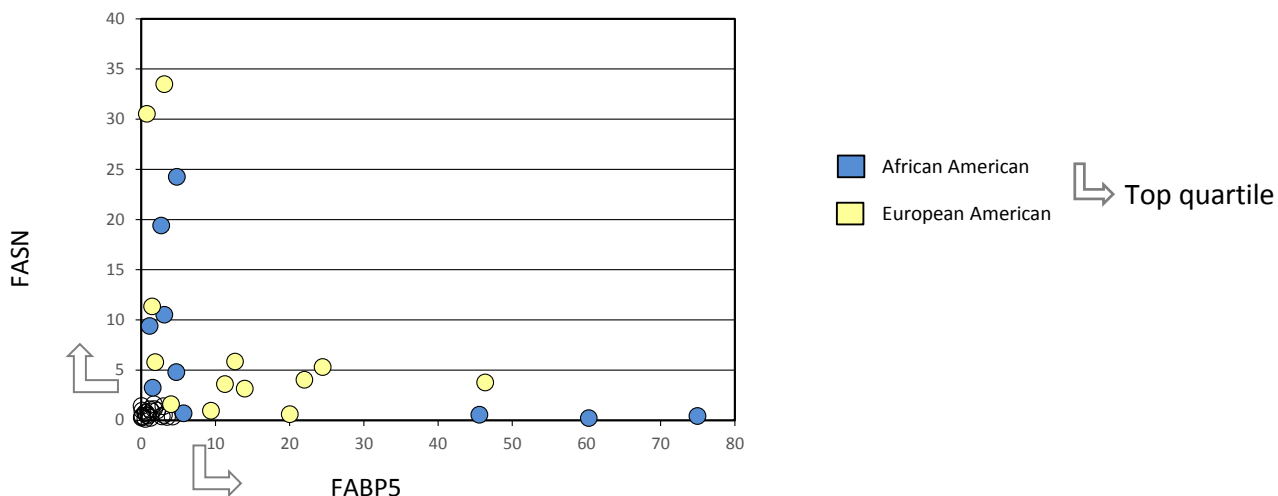
GE Figure 2: Super-overexpression (10 fold or greater) may define two PrCa subgroups with different molecular phenotypes for fatty acid processing/synthesis. Top quartile mRNA expression patterns for FABP5 and FASN in biopsies from AA and EA subjects are consistent with two PrCa subtypes that show an "either-or" super-overexpression at the mRNA level. In our study subjects, AA prostate cancers predominate in the subgroup showing the highest levels of FABP5 overexpression and EA prostate cancers in the subgroups showing the highest levels of FASN overexpression.

APPENDICES

<u>Cumulative Publications Resulting from this DOD Grant</u>: (Manuscripts were submitted in a timely fashion with previous reports)

A. Zhang HG, **Grizzle WE**. The effects of exosomes and related vesicles on cancer development, progression and dissemination. *In: Emerging Concepts of Tumor Exosome-Mediated Cell-Cell Communication,* (Eds HG Zhang), Springer Science. 2012;107-129.

B. McNally LR, Manne U, **Grizzle WE**. Post-transcriptional processing of genetic information and its relation to cancer. Biotech Histochem 2013; 88(7):365-72. **(This was in press during the last report. A final version of the published manuscript is included).**

C. Srivastava S., **Grizzle WE**. Biomarkers and the genetics of early neoplastic lesions. Cancer Biomark 2011;(9)(1-6):41-64. NIHMSID:NIHMS402044.

D. **Grizzle WE**, Srivastava S, Manne U. The biology of incipient, pre-invasive or intraepithelial neoplasia. Cancer Biomark 2011;9(1-6):21-39.

E. **Grizzle WE**, Srivastava S, Manne U. Translational pathology of neoplasia. Cancer Biomark 2011;9(1-6):7-20. NIHMSID: NIHMS402047.

F. **Gaston SM,** Guerra AL, Grooteclaes M, Renard, I, Kearney MC, Bigley J and Kearney GP. Gene Methylation Biomarker Analysis of Prostate Biopsies from Men with 1 of 12 Cores Positive for Cancer: Greater Methylation Prevalence and Extent in Gleason 7 than Gleason 6 Cancer. American Urological Association Annual Meeting, Washington DC; May 2011.

G. **Gaston SM**, Kearney GP, **Grizzle WE**. Prostate biopsy tissue print technologies; a practical and innovative approach to overcoming racial disparities in the datasets used for prostate cancer biomarker development. Presented at the AACR Cancer Health Disparities Meeting, Washington, D.C., September 18, 2011.

H. Otali D, He Q, Stockard CR, **Grizzle WE**. Preservation of immunorecognition by transferring cells from 10% neutral buffered formalin to 70% ethanol. Biotech Histochem 2013; 88(3-4):170-80.

I. Jones J, **Grizzle W**, Wang H, Yates C. MicroRNAs that affect prostate cancer: emphasis on prostate cancer in African Americans. Biotech Histochem 2013; 88(7):410-24.

J. **Grizzle WE**, Sexton KC, Bell WC. Human Tissue Biorepository. *In: Molecular Genetic Pathology*, volume 1, 2nd edition. (Eds. L Cheng, D. Zhang, Eble J), Springer Science+Business Media. 2013;483-497.

K. Bledsoe MJ, **Grizzle WE**. Use of Human Tissue in Research: The Current US Regulatory, Policy, and Scientific Landscape. Diagnostic Path 2013;19(9):322-330.

L. Zhang HG, **Grizzle WE**. Exosomes: a novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions. Am J Pathol 2014;184(1):28-41.

N. **Gaston SM**, Hayek JE, Otto G, Yen J, Bigley J, Neste LV, Kearney GP. Epigenetic field effect markers are indicative of occult high grade prostate cancer. Presented at the 2013 ASCO Genitourinary Cancers Symposium.

O. **Grizzle WE**. It is primarily the control of transcription and post-transcriptional processing that are critical to the development and progression of sporadic neoplasias. Biotech Histochem 2013; 88(7):361-4.

P. Theodore SC, Davis M, Zhou F, Wang H, Chen D, Rhim J, Dean-Colomb W, Turner T, Ji W, Zeng G, **Grizzle W**, Yates C. MicroRNA profiling of novel African American and Caucasian prostate cancer cell lines reveals a reciprocal regulatory relationship of miR-152 and DNA methyltranferase-1. Oncotarget Advance Publications 2014; 5(11):3512-25. PMCID:PMC4116499.

Q. Auer H, Mobley J, Ayers L, Bowen J, Chuaqui R, Johnson L, Livolsi V, Lubenski I, McGarvey D, Monovich L, Moskaluk C, Rumpel C, Sexton K, Washington M, Wiles K, **Grizzle W**, Ramirez N. The effects of frozen tissue storage conditions on the integrity of RNA and protein. Biotech Histochem 2014;89(7):518-528.

R. Van Neste L, Van Criekinge W, Bigley J, **Grizzle WE**, Adams GW, Karney GP, **Gaston SM**. To be presented at the EAU Section of Urological Research (ESUR) meeting in October 9-11, 2014, Glasgow, Scotland.

Abstracts /Presentation /Book Chapter in the reporting period (Included in appendix if abstracts are available)

S. The abstract "Limitations of the use of human prostate tissues in biomedical research" was presented by Dr. Grizzle at the Prostate Cancer Foundation 21st Annual Scientific Retreat, Carlsbad, CA, October 23, 2014.

T. The abstract "Performance of an epigenetic assay to predict prostate cancer aggressiveness: Comparing Gleason score and NCCN risk categories" was presented at the EAU Section of Urological Research (ESUR) meeting in October 9-11, 2014, Glasgow, Scotland.

U.   Dr. Grizzle was one of 5 presenters (Barnes M, Bledsoe MJ, Dressler L, Grizzle WE, Russell-Einhorn M) and panel participants in the all-day pre-meeting conference "Contemporary Issues in Biobanking: Governance, Consent and Practical Approaches to Current Challenges." This proceeded the conference "Advancing Ethical Research" of the organization, Public Responsibility in Medicine and Research (PRIM&R), Baltimore, MD, December 4, 2014.

V.   An abstract "DNA hypermethylation field effects: potential applications for detection of occult high grade prostate cancer" was presented at the 2015 Genitourinary Cancers Symposium of ASCO on February 26, 2015.

W.   An invited oral presentation on "Tissue Print Technologies for the Preparation of High Quality HumanBiospecimens" was presented by Dr. Gaston at the SELECTBIO Sample Preparation and Analysis Technologies conference in Boston, Massachusetts, March 2015.

X.   The abstract "Epigenetic Assay Stratifies Prostate Cancer Patients' Risk" was presented by Dr. Gaston at the 2015 American Urological Association Annual Meeting in May 2015.

Y.   An invited podium (oral) presentation "The use of innovative prostate biopsy tissue print techniques for molecular genomic, epigenetic and gene expression studies" was given by Dr. Gaston at the Minorities in Cancer Research Scientific Symposium "Emerging Methodology and Tools for Understanding the Genetics of Cancer Disparities" at the 2015 Annual Meeting of the American Association for Cancer Research.

Z.   An invited oral presentation "The Use of Tissue Prints of Prostate Cancer Biopsies for the Analysis of Non-Resected Prostate Cancer" was presented by Dr. Gaston at the Illumina Key Opinion Leader Biobank Summit in Boston, MA, May 2015.

AA.   An invited oral presentation on "Tissue resources and the Association of Racial Admixtures and the Risk for High Grade Prostate Cancer" was presented by Dr. Grizzle at the Illumina Key Opinion Leader Biobank Summit in Boston, MA, May 2015.

AB.   Abstract accepted entitled "Improving the accuracy and diagnostic power of prostate biopsy for African American patients: the Birmingham Alabama Prostate Cancer (BAPrCa) Consortium" to be presented by Dr. Gaston at the Eighth AACR Conference on the Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved in Atlanta, GA, November 2015 (copy attached to this report)

AC.   Abstract presentation entitled "Combined DNA-Methylation Intensity and Clinical Risk Score Stratifies Patients for High-Grade Disease" at the EAU Section of Urological Research (ESUR) meeting in Nijmegen, The Netherlands, September 2015 (copy attached to this report).

AD.   Invited oral presentation entitled "Tissue print technologies: An innovative and practical approach to obtaining high quality research samples from biopsies and other challenging biospecimens" will be presented by Dr. Gaston at IIR's Biorepositories and Sample Management Summit in Boston, MA, October 2015 (copy of the abstract for this presentation attached to this report)

AE.   Invited submission of FY15-FY16 PCRP program materials describing this DOD sponsored project; these materials include text and images for the program booklet, CDMRP website features, and the PCRP newsletter (PCRP Perspectives). September 2015 (copy of the invitation is attached to this report).

AF.   Book Chapter in Press

      Burke HB, Grizzle WE. Clinical Validation of Molecular Biomarkers in *Translational Medicine in Biomarkers in Cancer Screening and Early Detection*, Sudhir Srivastava, editor, Wiley, Oxford, UK.

Title: Improving the accuracy and diagnostic power of prostate biopsy for African American patients: the Birmingham Alabama Prostate Cancer (BAPrCa) Consortium

Sandra M. Gaston[1]; Soroush Rais-Bahrami[2]; Rick Kittles[3]; Kerry Dehimer[1]; Dennis Otali[2]; Jeffrey W. Nix[2]; Peter N. Kolettis[2]; George Adams[4]; William E. Grizzle[2].
[1]Tufts Medical Center, Boston, MA, [2]University of Alabama at Birmingham, Birmingham, Alabama, [3]University of Arizona, Tucson, Arizona, [4]Urology Centers of Alabama, Homewood, Alabama.

Study Purpose: Both incidence and mortality data show that the burden of prostate cancer (PrCa) is greater in African Americans (AA) than in European Americans (EA). Socioeconomic factors contribute to this health disparity, but do not fully account for observations that AA are more likely than others to be diagnosed with more aggressive and life threatening forms of PrCa. Prostate biopsies usually establish the diagnosis of PrCa and are used to estimate the extent of the disease (based on the number and location of cores with cancer and involvement of individual cores) and its potential aggressiveness (based on Gleason scores). Health policy groups recommend that men with limited low grade prostate cancer be managed by active surveillance (AS) rather than immediate surgical or radiation treatment. However, the standard-of-care prostate biopsy is limited by sampling error and the possibility that a high grade PrCa might have been missed is a significant concern for many patients who are considering AS; this concern is heightened for AA because of their higher risk of aggressive disease. Moreover, AA are more likely to be diagnosed with high grade/high stage prostate cancer that is not treated surgically and thus not well represented in molecular studies that utilize radical prostatectomy specimens. Our research team established the Birmingham Alabama Prostate Cancer (BAPrCa) Consortium with a major focus on the molecular analysis of prostate biopsies in order to increase the clinically actionable information that can be obtained from these specimens. We use an ancestry-informed approach that is specifically designed to improve the accuracy and diagnostic power of prostate biopsy for AA patients.

Experimental Procedures: The BAPrCa Consortium implemented an innovative prostate biopsy "tissue print" technology that permits collection of snap-frozen nitrocellulose blots of biopsy cores without diagnostically compromising these specimens. Tissue prints provide high quality RNA and DNA from biopsies from the full range of patients, including AAs whose cancer is too advanced at diagnosis for radical prostatectomy; this permits the molecular characterization of PrCa subtypes in men diagnosed with high volume/high grade disease who have not been adequately represented in previous molecular profiling studies. Our BAPrCa research protocols include informed consent for genetic ancestry admixture studies. Gene expression analysis of prostate biopsy tissue prints is correlated with histopathology and multiparametric prostate MRI.

Results: Our data suggest that in the Birmingham area, higher prostate cancer risk in AA is associated with increasing proportion of West African (WA) ancestry, which may reflect the prevalence of population-specific genetic mutations or variations that contribute to the development of more aggressive disease. As a group, the men diagnosed with high grade PrCa showed a significantly higher level of WA ancestry than the men diagnosed with no cancer (P = 0.001). A similar pattern is observed in comparisons of AA men diagnosed with high grade cancer vs low grade PrCa. Inasmuch as our AIMs genotyping panel uses a small

number of well-established AIMS markers, our observation of significantly increased risk of PrCa in AA men with high %WA AIMS ancestry may, if confirmed, have immediate potential clinical applications for improving prostate cancer screening and active surveillance. Moreover, gene expression profiles of biopsies from BAPrCa patients diagnosed with high volume/high grade PrCa revealed two subtypes of high grade PrCa with striking differences in the pathways that drive a shift in tumor fatty acid metabolism; one is a fatty acid synthase (FASN) dominant phenotype and the other a previously unrecognized fatty acid binding protein (FABP5) dominant phenotype. Our data suggest that the FABP5 dominant PrCa subtype is more common in AA and the FASN dominant subtype more common in EA. These findings may provide the basis for more effective dietary interventions and targeted therapies for AA and EA patients with high grade PrCa.

Conclusions: By utilizing innovative tissue print techniques for the molecular analyses of prostate biopsies and using an ancestry informed approach in our study designs, the Birmingham Alabama Prostate Cancer (BAPrCa) Consortium has identified new and potentially actionable PrCa signatures that may improve the accuracy and diagnostic power of prostate biopsy for AA patients.

Category - Genitourinary Cancers

Keywords - African Ancestry; Ancestry Admixture; Prostate Cancer Aggressiveness

Abstract #158029

Combined DNA-methylation intensity and clinical risk score stratifies patients for high-grade disease.

Leander Van Neste, Grant Stewart, Sandra Marlene Gaston, William E. Grizzle, George W. Adams, Gary P Kearney, Jonathan I Epstein, David James Harrison, Alan W. Partin, Wim Van Criekinge; Maastricht University Medical Center, Maastricht, Netherlands; University of Edinburgh, Edinburgh, United Kingdom; Tufts Medical Center, Boston, MA; Department of Pathology, University of Alabama at Birmingham, Birmingham, AL; Urology Centers of Alabama, Urology, Homewood, AL; New England Baptist Hospital, Boston, MA; Johns Hopkins University School of Medicine, Baltimore, MD; University of St. Andrews School of Medicine, St. Andrews, United Kingdom; The Johns Hopkins Hospital, Baltimore, MD; University of Ghent, Ghent, Belgium

Abstract Text:

Background: Prostate cancer (PCa) diagnostics remains challenging due to fear of over-diagnosis and overtreatment. Due to low accuracy of PSA too many men are biopsied that do not have a subsequent PCa diagnosis or that have indolent disease. Furthermore, persistent risk factors and fear of missed PCa leads to many unnecessary repeat biopsies. Most prostate tumors have epigenetic DNA-methylation aberrations, which display a field effect that can be observed in normal-appearing surrounding tissue, and that could help alleviate biopsy-sampling errors. **Methods:** A training cohort of methylation-positive men with a negative index biopsy followed by either a Gleason score (GS) ≥ 7 (n=43) or cancer-negative (n=226) repeat biopsy was evaluated. Using the initial negative biopsy, men were stratified for the likelihood of harboring high-grade PCa focusing on a methylation intensity algorithm involving *GSTP1*, *RASSF1* and *APC*. This algorithm was validated in a cohort of 102 men, with either a PCa-free (n=20), GS6 (n=46), or GS≥7 (n=36) biopsies. **Results:** The methylation intensity-based algorithm was developed on PCa-negative index biopsies and optimized to predict the presence of GS≥7 cancer in a repeat biopsy. The methylation intensity was significantly higher in GS≥7 compared to PCa-free repeat biopsies (p<0.001). Men with GS6 PCa detected upon repeat biopsy exhibited intermediate intensities. When combined into one model with clinical risk factors (age, pathology, DRE, PSA), an area under the curve (AUC) of 0.762 was obtained, which was significantly higher than the AUC of PSA (0.574; p=0.004) or the AUC of the clinical risk as calculated by the PCPT risk calculator (0.618; p=0.029). In the validation set, an AUC of 0.818 was obtained, with higher intensities for men with GS≥7 disease compared to men with GS6 PCa (p=0.002). **Conclusions:** The risk score can identify clinically significant cancer in PCa-negative biopsies and is strongly correlated with the GS of PCa-positive biopsies. The risk score could better stratify men for the need for repeat biopsy and the risk of harboring occult clinically significant PCa. The same algorithm could be used to segregate likely under-graded men from active surveillance candidates.

Title: Combined DNA-methylation intensity and clinical risk score stratifies patients for high-grade disease.
Submitter's E-mail Address: l.vanneste@maastrichtuniversity.nl
Is this a late-breaking abstract? No
Is this abstract a clinical trial? No
Would like to be considered for a Merit Award: No
First Author Membership: Non-Member

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EVALUATION OF RACIAL DISPARITIES ON PROSTATE CANCER DETECTION ON MRI/US FUSION-GUIDED PROSTATE BIOPSIES

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Objectives: Significant racial disparities exist between African American (AA) and non-African American men in Gleason Grade at the time of prostate cancer (PCa) diagnosis. To better characterize this disparity we used multiparametric magnetic resonance imaging (mpMRI) and targeted biopsies as a tool to assist in PCa detection.

Methods: Between January 2014 and August 2015, 177 patients who underwent mpMRI and MRI/ultrasound (US) fusion guided prostate biopsy and concurrent 12-core biopsy were reviewed. They were stratified by race but also protocol entry criteria: (1) prior negative prostate biopsy, (2) active surveillance protocol, or (3) primary biopsy evaluation for abnormal DRE or elevated PSA. MRI studies with T2-weighted, diffusion weighed, and dynamic contrast enhancement sequences were evaluated and areas of suspicion were identified. Patients underwent MRI/US fusion biopsies of targets and concurrent standard 12-core biopsy. The number of targets with PCa, number of standard biopsies with PCa, grade identified, and distribution of tumors was calculated.

Results Obtained: In our study, 38 AA males and 139 non-AA males underwent MRI/US fusion biopsies. PSA, age, and cancer detection on standard biopsy were not significantly different between groups. AA and non-AA men had a mean of 2.58 and 2.74 targets identified, respectively(p=N.S). The efficacy of targeted biopsy vs standard biopsy in detection of PCa and higher grade disease was equivalent between AA and non-AA males(p=N.S.). When both targeted cores and standard cores found PCa, standard cores in AA males showed higher grade PCa than targeted cores (p<0.001).

Conclusions: African American males have been shown to have higher risk of PCa and higher grade disease, but in our patient cohort undergoing MRI/US fusion−guided biopsy, cancer detection stratified by grade was equivalent. In patients with PCa found on both standard and targeted biopsy techniques, AA patients had higher grade disease on standard biopsy cores, likely a result of the distribution of AA patients referred with already diagnosed PCa on AS, suggesting a selection bias favoring the posterior peripheral zone location of their tumors.

	Total Population	African American	Non−African American	p-value
Number of Patients (%)	177	38 (21.5%)	139 (78.5%)	-
PSA (ng/ml), mean	9.59	9.7	9.57	0.67
Age (years), mean	63.3	62.8	63.3	0.41
Prostate Cancer Detection				
- No Cancer	79 (44.6%)	20 (52.6%)	59 (42.4%)	
- Cancer on Standard Cores Only	20 (11.3%)	6 (15.8%)	14 (10.1%)	0.33
- Cancer on Targeted Cores Only	37 (20.9%)	5 (13.2%)	32 (23.0%)	
- Cancer on Both Standard and Targeted Cores	41 (23.2%)	7 (18.4%)	34 (24.5%)	
- Higher Grade on Standard Cores		4	1	
- Higher Grade on Targeted Cores		8	13	0.001
- Equal Grade on Standard and Targeted Cores		1	20	
Highest Gleason Grade on Standard Cores				
0	104	25 (65.8%)	90 (64.7%)	
6	37	6 (15.8%)	26 (18.7%)	0.96
7	23	6 (15.8%)	19 (13.7%)	
8 or more	5	1 (2.6%)	4 (2.9%)	
Highest Gleason Grade on Target Cores				
0	99	26 (68.4%)	73 (52.5%)	
6	27	6 (15.8%)	21 (15.1%)	0.23
7	39	5 (13.2%)	34 (24.4%)	
8 or more	12	1 (2.6%)	11 (7.9%)	

Clinical Validation of Molecular Biomarkers in Translational Medicine

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Translational Relevance

Biomarkers are used in early detection, diagnosis, prognosis and risk assessment, in predicting responses to specific therapies and in evaluating therapeutic/preventive approaches (surrogate endpoints). Before biomarkers can be used clinically, they must be validated; however, in cancer there are few validated biomarkers for any of the above uses. Validation is a process that is not well understood by investigators and frequently biomarkers are described as validated when they have only begun validation. This manuscript describes and discusses a well-defined pathway with the steps that are necessary for validation of a biomarker for a specific use over a defined interval of time. This manuscript will aid investigators in the validation of their biomarkers, will clarify approaches needed for validation and will reduce the waste of resources for biomarkers that appear to be not strong enough to be validated for a specific use.

Abstract

Molecular biomarkers are required for improving the assessment of risk of disease, establishing the existence of disease, determining prognosis and treatment, and the implementing personalized medicine, and their clinical validation is a key step in translational medicine. Although many published papers claim to report clinically useful prognostic biomarkers, there are embarrassingly few validated cancer prognostic biomarkers. There are many reasons for this situation, one of which is that researchers may not fully appreciate the subtleties of molecular biomarkers and may not follow the rigorous procedures that are necessary to translate basic scientific findings to the clinic. We propose a straightforward approach to validating a biomarker using a well-defined, three-stage method. The stages are: 1) identification, characterization, and evaluation, 2) data and model testing, and 3) independent prospective replication of results. Also discussed are several important issues affecting the validation of biomarkers such as statistical model stability, the definition of clinical events, and combining molecular biomarkers into signatures and pathways. The goal of this manuscript is to clarify the process of validation and to provide guidance to investigators performing translational biomarker research.

Introduction

Molecular biomarkers are required for improving the assessment of risk of disease, establishing the existence of disease, determining prognosis and treatment, and the implementing personalized medicine (1-2), and their clinical validation is a key step in translational medicine (3-6). Validation is a rigorous process that requires a deep understanding of molecular biomarkers and their relationship to disease and an appreciation of the complexities inherent in their identification, testing, and replication (4).

In the past twenty years there has been an exponential increase in molecular biomarker research with thousands of new gene and protein biomarkers reported each year. At last count there are over five hundred thousand papers indexed in PubMed for gene, protein, and molecular biomarkers. Although many of these papers claim to report clinically useful prognostic biomarkers, there are embarrassingly few validated cancer prognostic biomarkers (7-10). There are many reasons for this situation (8-10), one of which is that researchers may not fully appreciate the subtleties of molecular biomarkers and may not follow the rigorous procedures that are necessary to translate basic scientific findings to the clinic (8-10). The result is studies replete with errors and a literature that contains incorrect, and many times even contradictory results (8-10). Because biomarkers are central to translational medicine, a failure to properly understand, assess, and utilize them has prevented their use in treatment, comparative benefit analyses, and in integrating individualized patient outcomes in clinical decision-making (8-11).

The validation of molecular biomarkers has been a concern since the earliest days of molecular research. Over the last twenty years significant problems have been noted, and recommendations regarding solving these problems have been made (12), but few of these proposals have been adopted. Pepe *et al.* (13) proposed a model for clinical validation of biomarkers for the early detection for disease, yet subsequent publications on early detection suggest that the confusion did not recede after this publication (14-17).

This manuscript proposes a straightforward, general method for validating biomarkers to assist investigators in their validation of molecular biomarkers. Because of the inherent complexity in analyzing biomarkers and the dynamic nature of the field, commentaries and general guidelines are provided.

Validation of Biomarkers

A molecular biomarker can be said to have been validated if it has been shown in an independent prospective replication study to reliably and accurately predict a specific outcome in a specified patient population over a defined time interval (1,3-4). At a minimum, a validated biomarker consists of a set of necessary and sufficient characteristics that uniquely identify the biomarker and includes the following: a detection and analysis protocol that results in high inter-laboratory agreement, a defined target patient population, a trained statistical model, i.e., a model whose parameters have been defined by the data that contains the biomarker, other relevant factors and the outcome of interest, and a quantitative statement of the accuracy of the biomarker at predicting the outcome of interest in the target population over the specified time interval (1,4).

For the purposes of this discussion, "molecular" refers to any sub-cellular factor, including proteogenomic, transcriptional, and metabolic factors (18). "Biomarker" refers to both individual and combinations of biological factors, including panels, patterns, profiles, pathways, and signatures that are used to predict one of three outcomes, namely, risk of disease, the existence of disease, and prognosis (1). There are three types of prognostic biomarkers, defined in terms of their use, namely, natural history, which predicts the course of the disease if the patient never receives a therapy; therapy-specific, which predicts whether a particular therapy will benefit the patient; and post therapy, which predicts that the therapy the patient received benefited the patient (4). "Outcome" is the clinical event of interest, e.g., incident disease, response to therapy, recurrence, or death. Although knowledge of the biological function of a molecular biomarker can provide important basic science information, functional information is not necessary for using a biomarker to predict a clinical outcome (2).

Predictive accuracy refers to the relationship of a predicted value to a true value for each patient, across a population of patients. It has two components, discrimination (the correct ordering of the predictions) and calibration (how close the predicted value is to the true value). A very useful measure of discriminative accuracy is the receiver operating characteristic (ROC) method (19-21). We are less interested in the calibration of the model than the correct ordering of the predictions because poorly calibrated models can be corrected by performing a post-processor calibration (22) but there can be no recovery from the low accuracy of a model that poorly discriminates.

Three Stages of Validation

We propose three stages to biomarker validation: 1) identification, characterization, and evaluation, 2) data and model testing, and 3) independent prospective replication of results (Table 1) (4). Each stage must be successfully completed before moving to the next stage. Prior to beginning the clinical validation process the investigator should be satisfied that the biomarker has the potential to answer an important clinical question. In other words, does the biomarker appear to be related to the disease, does the relationship appear to be very strong, and could use of the biomarker have an impact on patient outcomes?

For diseases in which the outcomes are easily predicted, no additional biomarkers are needed, and for diseases where there is no effective treatment, biomarkers will have little clinical utility. Additionally, the investigator should consider whether the biomarker is suitable for clinical use; in other words, is it relatively easily acquired and analyzed, is the analysis reproducible across laboratories, and is the acquisition and analysis of the biomarker relatively inexpensive. Further, will the biomarker be applicable to a sufficiently large number of patients so that its validation will make a clinical difference? Finally, the candidate biomarker should be examined in terms of whether it could add predictive accuracy when used with to the current biomarkers and whether it could eliminate one or more of the currently used biomarkers. If it neither adds predictive accuracy nor eliminates a current biomarker, then it is probably unnecessary. If the researcher believes that the evidence suggests that all these issues will be resolved in favor of the biomarker, then the validation process should proceed.

The validation of molecular biomarkers should progress through three stages (4). Stage 1 has three components: identification of the biomarker, characterization of the biomarker in terms of its specimen acquisition and analysis in its target population, and creation and evaluation of a multivariate supervised learning statistical model to determine the predictive power of the biomarker for a specific outcome over a specified time interval.

During stage 1, the investigator learns about the biomarker, including assessing the practicality of the acquisition of the biological specimen, its accuracy in various clinical populations, trying different statistical methods, determining a threshold (cut-off point for a continuous variable) for the biomarker, and examining the effects of confounders on the biomarker's accuracy. Most of the theoretical, biological, and experimental work related to the clinical validation of a molecular biomarker occurs in the first stage and the determination is made in this stage as to whether the biomarker is sufficiently accurate so that is warrants proceeding to the next stage in the validation process.

Stage 2, data and model testing, takes the final results of the first stage and attempts to implement and test them on another independent dataset from a different institution. This is an important stage because it reveals many of the unrecognized assumptions and biases that existed in the first stage. Stage 3, replication of results, is the critical stage since the clinical utility of the biomarker is established in this stage. Until the biomarker successfully completes the third stage, which requires an independent investigator, independent laboratory, and independent prospectively collected patient population, there is insufficient evidence that it can be applied to an important clinical problem.

Stage 1, Identification, Characterization, and Evaluation (ICE): In the ICE stage, the investigator selects and assesses a biomarker. There is no restriction as to how the biomarker is discovered. One of the first steps in identifying a potential risk or diagnostic biomarker is to determine if

the biomarker is expressed differentially in diseased versus non-diseased tissue, in other words, is it specific to the disease? The next step is to assess the biomarker's relationship to an outcome, i.e., risk of disease, diagnosis, or prognosis (1). Overall, there should be some evidence that the biomarker, as measured in solid tissue or in bodily fluids, is associated with the clinical outcome.

At the completion of this stage the biomarker should be described in sufficient detail so that it can be unambiguously and reproducibly identified and measured by other investigators (including the acquisition, storage and analysis of the biological specimen), its analysis is documented, a disease is specified, a clinical population relevant to the biomarker is identified, a disease-related outcome is selected, and the time interval during which the biomarker is relevant to the outcome is provided.

An example of a validated biomarker is the estrogen receptor (ER) of breast cancer (23). ER was initially described in terms of its measurement by radioimmunoassay, the specimen of malignant tissue and controls in which it was measured, the method of data analysis, the biomarker's relevance to a population of women with non-metastatic breast cancer, its disease related outcome, e.g., mortality due to breast cancer, and the time interval, e.g., 10 year disease-specific mortality, and better survival (24). The presence of a certain level of ER expression in the tumor predicts that anti-hormonal therapy will be effective in reducing women's' probability of a recurrence and of dying from their breast cancer (23,25).

Even though a single biomarker may be the primary focus of the validation, its clinical use will invariably rely on a multivariate model because the model must contain all the predictively relevant factors so that it can make accurate predictions (26-27). The goal of the model will be to contain all the independent, orthogonal predictors of the outcome. Further, the multivariate model will usually be related to an effective treatment, e.g., antihormonal therapy for ER expressing breast cancers, so that the biomarker predicts which patients will or will not respond to a specific therapy (28).

An initial approach to the analysis is to create a dataset containing patients to be analyzed for the biomarker and to randomly split the patients into training and testing subsets. The reason to spilt the data set is because the model developed on a single dataset will always have a high accuracy when it is assessed using the exact same patients on which it was developed. This high accuracy is due to over fitting and it reduces the model's generalizability. Therefore, the accuracy of the model should be determined on another dataset. It should be observed, that splitting the data is less than optimal because the training and testing data are subsets of the same patient population and contain the same biases. (We will discuss assessing the model's accuracy independent data sets.) The training subset determines the relationship between the independent and dependent variables and establishes that relationship in a statistical model. The test subset measures the accuracy of that trained model. For large data sets that contain many clinical (binary) events, e.g., dead/alive, recurrence/no recurrence, a fifty-fifty split is reasonable. For smaller data sets the more important component is the correct modeling of the disease phenomena, so more data is allocated to the training subset than the testing subset. A useful heuristic for small datasets is to split the data into two-thirds to three-fourths for training and one-fourth to one-third for testing (28).

The biomarker is modeled using an appropriate statistical method on the training dataset and its accuracy is tested on the testing dataset. During this stage, the investigator has knowledge of each patient's outcome and may examine the data, assess various statistical methods, add or remove biomarkers, and modify the analysis in any way. Various thresholds can be tested and the best one selected. There are no limitations on what may be done with the data or how the results are analyzed during this stage.

The discriminative accuracy of the model that contains the biomarker as a variable is measured on the testing dataset by the receiver operating characteristic (ROC). This is a critical juncture, for it is here that investigators can take a wrong turn. There is an inclination to believe that the results obtained on the ICE testing dataset have clinical meaning, but they do not because the investigator has optimized the biomarker, examined and manipulated the data and the analysis, looked at the results, and through trial and error, determined the best threshold, patient population, statistical model, and outcome for the biomarker. The biomarker's accuracy on an ICE dataset is not a valid measure of biomarker's clinical utility because this stage has the potential to produce overly optimistic and biased results. So far the

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investigator does not have valid results and neither the model nor its ROC developed in the ICE stage should be presented or published.

How to report studies of biomarkers used as prognostic factors is beyond the scope of this paper, however, other publications have addressed reporting prognostic biomarkers including REMARK, a checklist of 20 items (truncated to 11 items by some journal editors) that can be used to determine if a study of prognostic factors should be published (27,29-30), and STROBE-ME (31) that provides guidance on reporting observational molecular epidemiology studies.

Focusing on an understanding the scientific process regarding biomarker validation, which is the goal of this paper, can be more useful to an investigator than performing a study guided by whether it will meet a set of publication criteria. In other words, although problems with performing a study and problems with its publication can overlap, if the performance of a study is scientifically valid there should be few reporting problems, whereas, if the study is incorrectly designed and performed no publication guidance can save it.

Further, there are elements in some reporting approaches that may increase rather than decrease the quality and validity of publications on biomarkers. One example of this difficulty is the statement by Altman (27) that it is permissible to publish results after the investigator has looked at the data and used the resulting information to plan key features of the analysis to be perfomed using the same data. Our view is that data can only be looked at in the ICE stage, and then only with the understanding that the resulting ICE finding cannot be published.

Typically, the accuracy of a biomarker decreases as it progresses through the validation stages. At the end of the validation process it must retain sufficient accuracy to be clinically useful. In other words, the accuracy observed in the ICE stage will almost always be higher than the final validated accuracy of the biomarker. In order to save a biomarker investigator time and resources we suggest the following approach. A validated biomarker should have an ROC of at least 0.65 (assuming a standard deviation of 0.05 or less) to be clinically useful (2,32). Experience suggests that a biomarker will lose between 0.3 and 0.5 of its discriminative accuracy as it progresses thorough the stages of validation. Therefore, the minimum ROC for a biomarker to move from the ICE stage to the next stage should be 0.75. The relevance of these numbers will become more apparent in the clinical utility section of this paper.

Stage 2, Data and Model Testing (DMT): In this stage, the investigator uses the final characterization of the biomarker and statistical model derived from the ICE stage to test the biomarker. The researcher collects a new independent patient dataset (DMT dataset) from another investigator at a different institution (33). It includes the defined target patient population and appropriate biological samples for the measurement of the biomarker. The biomarker characteristics were determined based on the ICE study. The investigator then tests the ICE's final statistical model on the DMT dataset of patients. The critical component of this stage is the proper application of the final methods and results from the ICE stage to the new DMT patient population. The trained statistical model from the ICE stage can be tested *only once* on the DMT dataset. The DMT patients are run though the predictive model and the probability the outcome over the defined time interval for each patient is determined. The predicted outcomes are compared to the true outcomes and the predictive accuracy of the model is determined and reported in terms of the model's ROC on the DMT patients. The results must be sufficiently accurate to justify moving to the third stage of the validation process. In this case, the minimum ROC required to progress to the next stage is 0.70. If the biomarker does not achieve an acceptable accuracy in the DMT stage the investigator should determine if this failure was due to one of the following: the characterization and analysis of the biomarker, the statistical model, the characteristics of the patient population, the treatments included in the analysis, the conditions of the study, or other factors. The researcher can return to the ICE stage at any time, improve the biomarker or the model, and retest *once* on the DMT dataset. If the researcher uses the results of the DMT stage to improve the performance of the biomarker, then another independent patient population must be obtained for the DMT stage (labeled DMT2 dataset).

A successful evaluation of the biomarker does not mean that it has been validated because the same investigator who performed the ICE stage also performed its DMT stage and the dataset was

retrospectively created. At this point in the process many unknown and even unanticipated sources of bias may exist that can affect the power of the biomarker and the performance of the model, and therefore the accuracy and utility of the biomarker. For example, the investigator's method of dataset collection, biomarker analysis, and use of the statistical model are all subject to bias and error. Further, the datasets used in the first two stages are usually retrospective and subject to all the biases inherent in retrospective studies. Positive results of the DMT stage may be reported, but the report should contain the following explicit statement, "The reported biomarker results have not been validated and the biomarker is not ready for use in clinical practice." It is important that negative results of the DMT stage also be published (15).

Stage 3, Replication of Results (ROR): Because the hallmark of science is replication, a different investigator with a prospective, independently collected dataset should replicate the results of the DMT stage. This process is similar in approach to the DMT stage. The final model from the ICE stage, the one that was successfully used in the DMT stage, is applied only *once* to the ROR dataset. The model makes its predictions for the ROR patients and these predictions are compared to the true outcomes. If the ICE stage results were reproduced in the DMT stage but not in the ROR stage, this suggests that either there was a bias in the datasets used in one or more of the stages of the validation process or there were problems with the performance of the biomarker assay.

Clinical utility means that the biomarker improves the management and outcomes of patients (30). Determining the potential clinical utility of a biomarker is a complex concept (34). It includes, but is not limited to, the acquisition and analysis of the biomarker, the number of patients with the target disease, the severity of the target disease, the safety and efficacy of the treatment, and the accuracy of the test in predicting a therapy-specific benefit. Herein, the discussion of clinical utility is limited and only includes the accuracy of the biomarker. A necessary requirement for clinical utility is that the biomarker is significantly more accurate than chance prediction, i.e., an ROC of 0.50; thus the minimum biomarker accuracy of an ROC of 0.65, because lower accuracies are unlikely to surmount chance. It should be noted that accuracies of at least 0.70, and a standard deviation less than 0.05, are preferred. Increasing a low ROC requires either starting with a more powerful biomarker or reducing the variance of the predictions.

Issues Related to Validating Biomarkers

Biomarker Datasets: The datasets used for validation should include the current clinical predictive factors, the relevant confounders, and the effective treatments. They should have a sufficient number of patients and events for model stability (discussed subsequently), and the patients should be followed for a sufficient period of time, defined by the clinical problem the biomarker is addressing, so that the predictions are clinically meaningful.

Most biomarker studies are conducted using retrospective populations. These datasets have the advantages of being readily available with relatively long periods of follow-up, thus making them quick and much less expensive to acquire and use. The main disadvantages of retrospective data sets are: 1) they may contain biases associated with patient selection, or specimen acquisition and analysis, or treatment, 2) they usually do not contain all the relevant predictors and confounders, i.e., there can be unmeasured covariates, 3) they almost always contain heterogeneous patient populations and therapies, 4) not all the patients may have been assessed for the candidate biomarker (i.e., appropriate biological samples may not be available), 5) the therapies are not uniformly applied across patients resulting in a surprising number of different treatment regimens, 6) they may contain patients treated with antiquated therapies and/or inadequate numbers of patients may have been treated with current therapies, and 7) they typically contain a great deal of missing data which can make them unsuitable for multivariate analysis.

A key issue in retrospective data is the absence of biomarker values in some of the patients. The values could be missing completely at random but this is rarely the case (35). Usually a bias is at work. The investigator has a number of ways to deal with this problem, including only using the patients who have a biomarker value, imputing a central tendency biomarker value, or finding the specimens and

assessing the missing biomarker value. In terms of solving the missing data problem, finding the specimens and assessing the biomarker is usually the best approach. If this is not possible, then performing multiple imputation may be a useful alternative approach (36). In any event, if the missing biomarker values affect the validation results, for example, if there is an important bias at work in the data, it will be discovered in Stage 3 by a significant decrement in accuracy. Thus, retrospective studies are not definitive evidence of the accuracy and clinical utility of a biomarker.

Prospectively collected populations avoid many of the weaknesses inherent to retrospective studies. A prospective study follows a defined population, it collects all the relevant variables and samples, it implements uniform biomarker detection methods and therapy regimens, and the patients have been followed for a pre-specified period of time. The major limitations of prospective studies are: they have entry criteria that create a relatively homogeneous patient sub-population (in part to reduce patient variability), they require extensive financial and manpower resources, and they take a long time to complete. Further, they may not be generalizable to most patients with the disease because the patients in the study were a special sub-population, because of the study's tight clinical control, and because many patients in the real world will not receive the exact therapy offered in the trial. Due of the time and cost of prospective studies, retrospective studes are usually employed in the ICE and DMT stages, the results of which are used to justify the time and cost of a prospective replication study.

Implicit in this discussion is the knowledge that prospective datasets are usually collected to evaluate a specific therapy. Their use in the ROR stage is based on the idea that not all the patients who receive the therapy will respond and that this differential clinical effect can be used to define the utility of the biomarker in predicting which patients will respond to the therapy (therapy-specific prognosis) and predicting which patients, after receiving the therapy, responded to it by a change in the biomarker value (post-therapy prognosis) (1).

Statistical Model Instability: An important consideration in building statistical models is to avoid model instability. Model instability occurs when the relationship between the independent variable and the dependent variable is not linked strongly enough in the model. The result is that the model's parameter estimates vary over too great a range. It has been suggested that to avoid model instability there must be at least 10 events (defined subsequently) for each independent variable (37), however, for the analysis of the predictive power of molecular biomarkers 15 – 20 events provide a greater assurance of model stability. With this number of events, the relationship between each independent variable and the outcome can be reliably determined (to the extent that the independent variable is a strong predictor of the outcome). Alternatively, one can use the bootstrap method to test for model instability (38-39).

Clinical events: A clinical event is defined as the least frequent clinical outcome (4). Thus, for a binary outcome, e.g., alive or dead, whichever occurs *least* often is the event rate. The optimal event rate for the analysis of a binary outcome is 50%. As the event rate diverges from 50% toward 0% or 100% it becomes easier to make predictions because a model will predict that the more frequent event will always occur and it will be correct more and more of the time. For example, in terms of percent correct, if the event rate is 10% the model will be correct 90% of the time if it always predicts the occurrence of the non-event. In other words, statistical models can learn to ignore the independent variables and "bet on the frequency" (4). In fact, in clinical conditions with very low event rates, it is rarely possible for the independent variables to do as well as predicting the outcome as betting on the frequency. This illustrates why an analysis cannot be based on an accuracy measure such as percent correct. The ROC adjusts for the event frequency.

Combining molecular biomarkers: Although a detailed discussion of the acquisition and analysis of molecular factors for purposed other than biomarker validation is beyond the scope of this paper (40-41), there are certain issues related to the validation of these biomarkers that must be addressed. One can combine molecular biomarkers under various rubrics including panels, patterns, profiles, signatures, and pathways. The goal of combining biomarkers is usually to increase predictive power beyond that afforded

by an individual molecular biomarker. There are at least two approaches to combining molecular biomarkers. One approach can be called "naïve" because it groups biomarkers using a statistical algorithm that does not use any previously known information regarding the biomarkers or the relationships between the biomarkers. The idea is that the relationship between the biomarkers will become apparent through their statistical association.

Another approach, which can be called "functional," captures the power inherent in functionally related biomarkers, such as membership in a biological pathway that is related to the disease process. The idea is that there is prior scientific knowledge that certain biomarkers are related to each other and to the disease and this previous knowledge can be used to inform the statistical model. In other words, naive groupings make use the numerical information in the dataset but they ignore all other information, whereas functional groupings use the numerical information in the dataset and, in addition, they take advantage of previous scientific knowledge regarding relations among biomarkers.

A functional group can be any set of related biomarkers. There is no restriction on the composition of a functional group other than it must consist of factors that are related to the disease process. The idea is that a subset of the pathway factors will be active at any one time and thus are predictive of the course of the disease. Further, one would like to include multiple orthogonal pathways, i.e., each providing new information regarding the disease process, in order to further increase predictive power. Because of the multiplicity of molecular biomarkers that comprise a pathway, the functional approach can be an effective way to combine many related biomarkers. The biomarkers in the pathway can be integrated using partial least squares, principal components, or similar dimension-reduction strategies and the integrated biomarkers can be one variable in the multivariate statistical model. Thus, each orthogonal pathway can be represented as a variable in a multivariate model. Generally, functional groups, rather than individual biomarkers, have the greatest chance of being strongly predictive.

Sometimes investigators create a group of genes, e.g., 10 genes, and claim that this is a unitary gene signature and the genes are necessary and sufficient to be the signature for some outcome. But when the researchers attempt to reproduce their finding in another study, instead of reproducing the entire group of significant genes, they find that only 6 of the genes are significantly associated with the outcome in the repeat study. They may wish to claim that the 6 genes are now the validated gene signature. The problem is that the researchers cannot claim that the signature is composed of 10 genes when only 6 of the signature genes can be reproduced, nor can they claim that the combination of 6 genes is a new, replicated signature. Clearly, one cannot have it both ways, one cannot claim that there is a validated gene pattern when the pattern does not replicate completely or abandon the pattern for another pattern, yet claim that the original signature was replicated. On the other hand, in a functional group, when one claims that a related set of genes is the predictive unit of analysis, it is not expected that all the genes in the group will always be significantly over or under expressed.

One method for assessing the predictive power of a biomarker in a multivariate model is to remove the biomarker from the model and observe a change in predictive accuracy (28). In this approach each variable is, in turn, removed, assessed, and returned to the model. The idea is that if the biomarker is a powerful predictor a large decrement in accuracy will be observed when it is removed. It should be noted that this is a complex process since it also involves issues related to collinearly and levels of analysis. Analysis levels refer to the type of units being analyzed. For example, one can posit three levels of analysis in cancer, namely, epidemiologic, e.g., age, race, etc., anatomic and cellular, e.g., tumor size, histology, etc., and molecular-genetic, e.g., ER, PR, HER-2 (18).

Time denomination of the biomarker: Predictions, i.e., the probability of the occurrence or non-occurrence of an event, must always be time denominated (1,4). For example, the probability of an event occurring in five years is different than the probability of that same event occurring in ten years. There are two reasons why the prediction's duration must accompany its numerical estimate. First, time itself affects the probably of the outcome. For example, it may be more difficult to make predictions in the middle of the time interval (where the interval is bounded by the index date and the end of the study). Second, the biomarker may only be related to the disease ("active") at a particular time in the disease

process, rather than uniformly across the course of the disease. Thus, a biomarker may be useful in predicting an outcome at 2 years but not useful in predicting the same outcome at 10 years. In other words, when a biomarker makes a prediction, that prediction is only relevant for a defined population, a specific outcome, and over a specified period of time. Finally, lifetime predictions are rarely clinically useful because it is not clear what the duration of the patient's lifetime will be, therefore, the time interval of the prediction is unknown.

Conclusion

If we are going to model diseases in terms of their molecular characteristics, and these models are going to drive future advances in medical care, then translational science must produce clinically validated molecular biomarkers. Unfortunately, molecular biomarkers are subtle and complex entities, and their validation is challenging. Advances in the validation of clinically useful biomarkers requires an unambiguous scientific nomenclature, clearly described and defined methods, and clinically relevant uses if the molecular biomarkers are to significantly impact medical care. To minimize the reporting and use of biomarkers that cannot be validated a straightforward three-stage approach to biomarker validation is described. The three stages are: 1) biomarker identification, characterization and evaluation, 2) data and model testing, and 3) replication of results. This provides a scientific approach that, if followed, offers a high degree of certainty that a validated biomarker will be a true and clinically useful predictor of disease-related outcomes.

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Table 1. Characteristics of validation

Category	Stage 1	Stage 2	Stage 3
Investigator	Original	Original	Independent
Data	Retrospective or prospective	Different retrospective or prospective	Different prospective
Analysis	Any	Pre-specified	Pre-specified
Minimum Model Accuracy*	0.75	0.70	0.65
Reporting results	No	Qualified	Unqualified

*Minimum model accuracy is the discriminative accuracy of the statistical model that includes the biomarker and the biomarker adds significant predictive accuracy to the model.